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INVESTIGATIONS OF 167 BASE PAIR CORE PARTICLE STRUCTURE AND THE ROLE OF UBIQUITIN IN CORE PARTICLE STRUCTURE.

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CERTIFICATE OF SUPERVISORS

In terms of paragraph eight of "General regulations for the degree of Ph.D", we, as supervisors of the candidate N. H. Davies, certify that we approve of the incorporation into this thesis of material that has already been published or submitted for publication.

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ABBREVIATIONS

EDTA	ethylene diamine tetra-acetic acid
EGTA	ethyleneglycol-bis-(2-amino-ethyl ether) N, N' tetra-acetic acid
Tris	tris (hydroxymethyl)-aminomethane
ATP	adenosine 5' triphosphate
DNase 1	pancreatic deoxyribonuclease
MNase	micrococcal nuclease
TCA	trichloroacetic acid
BSA	bovine serum albumin
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
bp	base pairs
PMSF	phenylmethylsulfonyl fluoride
DTT	dithiothreitol
$NaBH_4$	sodium borohydride
Tween 20	polyoxyethylene sorbitan monolaurate

SUMMARY

The zero length protein-DNA crosslinking methodology developed by Mirzabekov and co-workers was established and successfully applied to a 167 bp core particle containing two full turns of DNA and no linker histones. The problems encountered in the methodology were examined in detail. The histone-DNA contacts in the 167 bp core particle are discussed with particular reference to the 146 bp core particle.

Proteins of higher molecular weight than the core histones were detected bound to the 3' end of both 146 bp and 167 bp core particles. One of these proteins was purified and partially sequenced. The partial sequence was identical to that of histone H2B indicating that the protein is some form of large histone H2B variant.

An attempt was made to investigate the involvement of the ubiquitinated histones, uH2A and uH2B, with active chromatin using a combination of the protein-DNA crosslinking and immunological methodology. Attempts to overcome the limitations of the antibodies raised against ubiquitin are detailed and discussed.

The effect of the incorporation of the ubiquitinated histones, uH2A and uH2B, into the core particle (using poly(glutamic) acid mediated assembly) was examined using MNase and DNase 1.

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CHAPTER 1

THE STRUCTURE OF THE CORE PARTICLE

1.1 Introduction

The fundamental element of chromatin, the nucleosome (two turns of DNA wrapped around the core histone octamer together with histone H1 and a variable length of linker DNA depending on the source) has been the focus of intensive investigation since the early 1970s. A history of the evolution of the nucleosomal model of chromatin is reported by van Holde (1989) and Kornberg and Klug (1981). Similarly, the core particle (one and three quarter turns of DNA wrapped around the core histone octamer) has been extensively reviewed (Kornberg (1977), Igo-Kemenes et al. (1982), McGhee and Felsenfeld (1980a), van Holde (1989)). Only a brief summary is therefore presented. The information gathered from diffraction studies will be presented in greater detail, however, as these have given the most comprehensive structural picture of the core particle. They are also particularly pertinent to the area of investigation reported in this thesis, the study of histone-DNA contacts in a two turn 167 bp core particle by means of a zero length histone-DNA crosslinking methodology established by Mirzabekov and co-workers (Mirzabekov et al. (1989)). The results of the crosslinking methodology and evidence for a two turn core particle will therefore be discussed in some detail.

1.2 The basic components of the core particle

Enzymatic digestion of chromatin or nuclei to monomeric particles proceeds through several discernible stages. A nucleosome is initially released consisting of 160-240 bp of DNA associated with one histone H1 and an octamer of core histones (H2A, H2B, H3 and H4) (Kornberg (1977)), McGhee and Felsenfeld (1980b)). Further digestion results in formation of the chromatosome (Simpson (1978a)) (160-170 bp of DNA associated with histone H1 and the core histone octamer

(Varshavsky et al. (1976))) followed by the fleeting appearance of a particle with 167 bp of DNA together with the core histone octamer (Weischet et al. (1979), Lindsey and Thompson (1989)). This is then proceeded by a more marked pause in digestion, the core particle with 146 bp of DNA plus the core histone octamer (Rill and van Holde (1973)).

The involvement of the histone octamer as the central protein core in the repeating unit of chromatin was deduced from chemical crosslinking studies on histone complexes in solution and in chromatin (Kornberg (1974), Kornberg and Thomas (1974), Stein et al. (1977)) and confirmed in the detailed study of Albright et al. (1979). In this investigation mouse tissue culture cells were double labelled with ^{14}C-lysine and ^{3}H-arginine and nuclease digestion products were then electrophoretically isolated. The histones associated with the nuclease digestion products were separated by SDS PAGE and their relative molar ratios accurately quantitated by scintillation counting. The molar ratios of the core histones were shown to be equal and combining this result with the molecular weight of the core particle only allows a octamer with two copies of each core histone.

The DNA component of the core particle has been accurately sized to 146 ±3 bp in a wide range of species (Lohr and van Holde (1979), Grellet et al. (1980), Shindo et al. (1980)). Neutron diffraction studies determined that the path of the DNA is around the outside of the histone octamer (Pardon et al. (1975)) and X-ray diffraction studies confirmed this finding (see below).

1.3 Physico-chemical studies of the core particle

The structure of the core particle has been investigated using many different techniques apart from X-ray and neutron diffraction studies. These include histone-histone and histone-DNA crosslinking, DNase 1 digestion, thermal denaturation and the study of subnucleosomal particles. As

mentioned above, histone-DNA crosslinking investigations are a major part of this thesis and therefore will be discussed separately in a later section.

1.3.1 Tetramer and dimer organisation of core histones

The chemical crosslinking and sedimentation studies (Kornberg and Thomas (1974)) also identified a H3-H4 tetramer and a H2A-H2B dimer. The histone octamer from the nucleosome can be isolated intact in 2M NaCl (Thomas and Kornberg (1975)); the octamer can reversibly dissociate with the loss of one H2A-H2B dimer (Godfrey et al. (1980)). The H3-H4 tetramer is generated by the dissociation of the second H2A-H2B dimer (Eickbush and Moudrianakis (1978)). When chromatin is exposed to an increasing concentration of salt, H2A and H2B are released at about 0.8 M NaCl and H3 and H4 at about 1.5 M (Burton et al. (1978)).

Support for the tetramer and dimers was also obtained from other crosslinking studies. As mentioned above, histone-histone crosslinking with dimethyl suberimidate was crucial for the establishment of the existence of the octamer, tetramer and dimer (Kornberg and Thomas (1974)), Stein et al. (1977)). The ability of dimethyl suberimidate to span distances of 10 to 20 Å reduces its ability to give information about the more immediate neighbourhood of each histone. Shorter or direct crosslinks have therefore been used in many studies. The results can be summarized as follows:

1. Heterotypic dimers are formed between all the core histones but those between H2A and H2B, H2B and H4, H3 and H2A have generally been observed most frequently (van Holde (1989), Isenberg (1979), Jackson (1978)).
2. Homotypic dimers are rarely observed except for the H3-H3 (Gould et al. (1980), Ausio et al. (1984), Jackson (1978)) and H2A-H2A dimer (Jackson (1978)).

The H2A-H2B crosslinks support the existence of the H2A-H2B dimer. The crosslink between H2B and H4 has been postulated to be present at the interface between the H2A-H2B dimer and the H3-H4 tetramer (Klug et al. (1980)). The homotypic dimer of H3 indicates that the tetramer is mainly held together by H3-H3 interactions (van Holde (1989)). It has been suggested that the H2A-H2A dimer is possibly the result of an internucleosomal crosslink (Burlingame et al. (1985)) since the individual H2A histones are well separated in the core particle.

1.3.2 Domains of histone-DNA interaction

Thermal denaturation of core particles reveals two different types of histone-DNA interaction. DNA exhibits an increase in absorbance at 260 nm (hyperchromicity) when heated due to denaturation of the double helix into single-stranded DNA. If core particles are heated, the hyperchromic curve is biphasic with about 30 % (42 bp) of the DNA melting at 58°C and the remainder at 74°C (Weischet et al. (1978)). Through the use of circular dichroism and calorimetric measurements, it could be deduced that the initial melt only involved DNA and not the histones. In the second melt the protein core and the remainder of the DNA denatured. It was proposed that the ends of the DNA melted in the first transition (Weischet et al. (1978)). This was addressed by Simpson (1979), who melted 5' ^{32}P end-labelled core particles. The core particles were reconstituted with the synthetic DNA, poly(dA.dT).poly(dA.dT), as this form of DNA has a low melting temperature allowing the first transition to occur at 40°C to 50°C. These cores were then digested with the single-stranded nuclease, exonuclease III, at 45°C and DNA with a length of 105 bp with no end label was produced. About 20 bp must therefore melt at either end of the core particle DNA and be exposed to digestion by exonuclease III. The salt dependence of the melting temperature of this first transition was analysed by McGhee and Felsenfeld (1980b). From this analysis it was estimated

that there are only three strong ionic interactions between the histones and each turn of the DNA helix.

Continued micrococcal nuclease digestion of the core particle results in the formation of several well defined subnucleosomal particles (Rill et al. (1975)), Nelson et al. (1982)), Bakayev et al. (1981), Domanskii et al. (1982)). Digestion of 5' ^{32}P end-labelled core particles released two particles SN4 and SN7 amongst others (Bakayev et al. (1981)). SN7 consisted of 108 bp of DNA and all the core histones except a H2A-H2B dimer whereas SN4 contained 40 bp of DNA and a H2A-H2B dimer. The total length of DNA associated with the core particle could be accounted for and moreover each particle contained a 5'-end label. Very similar results were obtained by Nelson et al. (1982)) with DNase 1 and micrococcal nuclease digestions. This study also obtained a particle with 70 to 80 bp of DNA and only a dimer of H3 and H4. This suggested that the H2A-H2B dimers are localized near the ends of the core DNA with H3 and H4 associated with the central region.

The H3-H4 tetramer is also implicated in having a central role in stabilizing the core particle. H3 and H4 are more tightly bound to the DNA than H2A and H2B (see above). A H3-H4 tetramer, reconstituted onto part of the 5S ribosomal RNA gene of Xenopus Borealis, organized 120 bp of DNA in an identical manner to that found in the core particle (Hayes et al. (1991)) and at higher H3-H4/DNA ratios an octamer of H3 and H4 protected a core length of DNA (Cammerini-Otero et al. (1977)).

1.4 Diffraction studies

Though the type of studies presented above (see also section 1.5) have yielded a great deal of information about the core particle, diffraction and scattering studies have been the most direct means of gaining three dimensional information. Since the first publication (Finch et al. (1977)) of a X-ray

diffraction and electron microscopy study at 20 Å resolution of proteolysed core particles, the resolution has been steadily improved and the discrepancies between the models of the various workers in the field reduced.

The initial model proposed by Finch et al. (1977) had general features very close to those currently accepted. It was suggested that the shape of the particle was a wedge shaped disc with a thickness of 57 Å and a diameter of 110 Å. The particle was found to be bifurcate with a dyad axis. The DNA was modelled to form a left handed superhelix which wound around the histone octamer in 1.75 turns with a superhelical pitch of about 28 Å. DNase 1 digestion studies of core particles (Noll (1974), Lutter (1978)) supported the superhelical nature of the core DNA and established the left handedness of the superhelix.

The histone octamer three dimensional structure was resolved to 22 Å using electron microscopy and image reconstruction methodology (Klug et al. (1980)). The histone-histone (see above) and the histone-DNA (see below) chemical crosslinking data were used to assign regions of density in the octamer map to specific histones. The octamer had two-fold symmetry and was wedge shaped with a 70 Å diameter and a maximum thickness of 56 Å. The H3-H4 tetramer occupied the central region and was flanked by the two H2A-H2B dimers. A rough left handed helical ramp with a pitch of 25 Å could be discerned on the face of the octamer suitable for the winding of 1.75 turns of the DNA superhelix.

The core particle structure has been resolved to 7 Å by Richmond et al. (1984) and to 8 Å by Uberbacher and Bunick (1989). The position of the histones is again assigned on the basis of density continuity within the map and chemical crosslinking studies. Richmond et al. (1984), however, confirmed the location of H3 with heavy atom labelling. The two models are generally similar but have some notable differences.

The DNA is wrapped around the histone octamer with about 1.8 left handed superhelical turns. There are approximately 7.6 helical turns per superhelical turn with a mean DNA repeat of 35 Å. The superhelical pitch varies between 25 and 30 Å and the average superhelical radius is 43 Å. The dyad axis passes through the middle of the core DNA where the minor groove faces outward. This position has been labelled 0 and each sequential turn of the DNA helix from this point to the ends by ±1 to ±7 (Klug et al. (1980)). The curvature of the DNA is punctuated by several sharp bends in the DNA at ±1 and ±4. These regions are maximally protected from DNase 1 cleavage and the helical repeat near ±1 is maximally accessible to dimethyl sulfate (McGhee and Felsenfeld (1979)) and to singlet oxygen (Hogan et al. (1987)).

Uberbacher and Bunick (1989) calculated the periodicity of the DNA to be 10.2 ± 0.05 bp. This is very close to the value of 10.17 bp determined by Drew and Travers (1985) using statistical sequencing. Drew and Travers used their value and the observed 7.6 double turns of DNA per superhelical turn in the core particle (Richmond et al. (1984), Uberbacher and Bunick (1989)) to resolve the "linking number paradox". This arose from the physicochemical measurements performed on the SV 40 minichromosome (Germond et al. (1975)) which suggested only one (rather than two) superhelical turn per core particle. As pointed out by Finch et al. (1977) and Klug et al. (1981), the change in linking number is derived from the sum of the number of superhelical turns plus any change in the local twist induced by the protein (Crick (1975)). If the local twist changes from 10.6 bp in free DNA (Rhodes and Klug (1980)) to about 10.0 bp per turn, the paradox would be resolved (Klug et al. (1982)). The calculated value of -1.25 arrived at by Drew and Travers agreed closely with the empirical value of -1 to -1.25 (Germond et al. (1975), Klug and Lutter (1980)).

There does not appear to be any meaningful amount of protein density extruding between the gyres. The H3-H4 tetramer occupies and binds to the central superhelical turn (positions -4 to +4). The two H3 histones lie symmetrically on either side of the dyad axis and bind extensively from -2.5 to +2.5. H4 makes substantial contacts out to ± 3.5 and minor contacts from ±4 to ±5.

Though both studies place the H2A-H2B dimers in a position to interact with DNA from ±4 to ±7, there are two significant differences. These are (1) Uberbacher and Bunick (1989) find the two dimers to be symmetrical and Richmond et al. (1984) find them to be asymmetrical and (2) Richmond et al. (1984) postulate that H2A by extending out over position 0 would block the DNA exit path. H2A is close to this region in the model of Uberbacher and Bunick (1989) but it does not appear to impede DNA egress. Uberbacher and Bunick (1989) suggested that these differences in the Richmond et al. (1984) study arose from packing forces and/or the use of 1,6-hexanediol. This suggestion has very recently, been directly addressed with the direct comparison of a core particle crystal that has been treated post growth with the alcohol (partially dehydrated) and one which has not (fully hydrated) (Struck et al. (1992)). The fully hydrated core particle at 9 Å resolution did show full symmetry of the H2A-H2B dimers but the DNA at position +7 was asymmetric relative to -7. H2A extends over the dyad axis in the hydrated core particle as it does in the partially hydrated core (Richmond et al. (1984)) and Struck et al. (1992) suggest that the difference noted in (2) above could be caused by differences in the methods employed to obtain the electron density map.

An X-ray crystallographic structure of the octamer at 3.3 Å resolution presented by Burlingame et al. (1985) resulted in considerable debate in the literature (Klug et al. (1985), Moudrianakis et al. (1985a), Uberbacher and Bunick (1985a), Moudrianakis et al. (1985b), Uberbacher et al. (1986)). The structure was reported as being a prolate ellipsoid 110 Å by

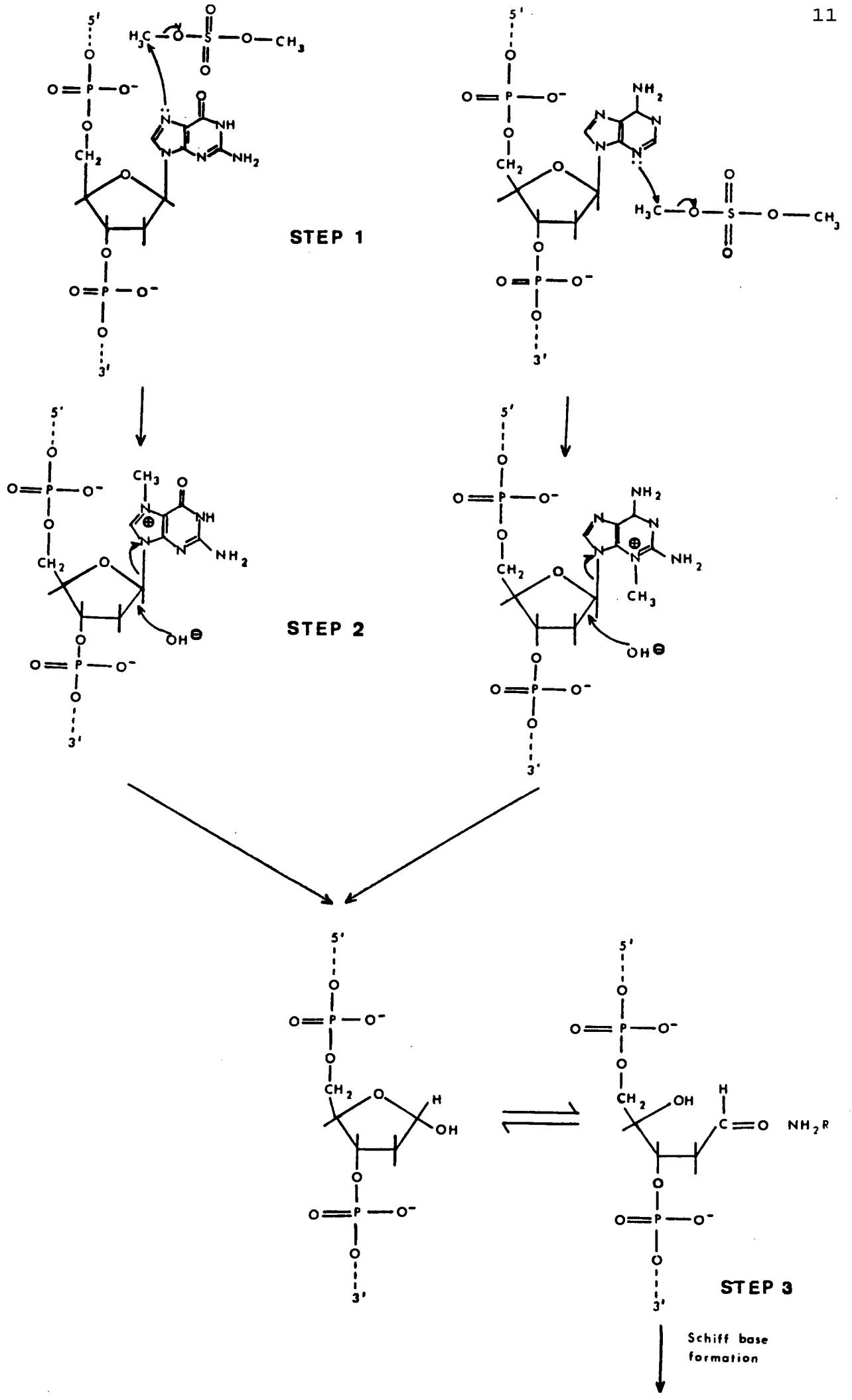
65-70 Å (Burlingame et al. (1985)). This differed dramatically from the dimensions and shape (wedge shaped, 70 Å diameter, 56 Å thickness) previously reported (Finch et al. (1977), Richmond et al. (1984), Klug et al. (1980), Uberbacher and Bunick (1985b)). The data for the 3.3 Å structure (Burlingame et al. (1985)) has recently been re-analysed by Moudrianakis and co-workers (Arents et al. (1991)) and found to be consistent with the wedge shaped structure. The stucture now presented at 3.1 Å resolution has a diameter of 65 Å, a length of 60 Å at its maximum and 10 Å at its minimum. At this high resolution they were able to directly identify the individual histones through assigning side-chain densities and did not have to rely on chemical crosslinking evidence. The model is very similar to that previously established with the H3-H4 tetramer being flanked by two H2A-H2B dimers. The tetramer consists of two crescent-shaped H3-H4 dimers which interact at the twofold axis to form the tetramer. The H2A-H2B dimers are similar in shape and size to the H3-H4 dimers. The helical ramp proposed by Klug et al. (1980) can be seen as a "left-handed proteinaceous superhelix" with a 28 Å pitch in close agreement with the pitch determined for the DNA superhelical pitch on the core particle (Richmond et al. (1984), Uberbacher and Bunick (1989)). The individual histones have a common structural motif (termed the "histone fold") in their central regions involving a long central α helix with a loop and a shorter helix on either side. The histones are folded in an elongated form rather than a single compact globular domain. Within each dimer (H3-H4 and H2A-H2B), the histones "interdigitate" rather than each histone occupying a single defined region in the octamer.

Higher resolution analysis of the core particle probably requires defined DNA (sequence and length) and homogeneous histones (Richmond et al. (1988)). Preliminary results using this approach have been reported by Richmond et al. (1988).

1.5 Histone-DNA crosslinking

A procedure to crosslink DNA to histones under mild conditions was developed by Levina and Mirzabekov (1975). The chemistry of the crosslinking method has been presented in detail (Levina et al. (1981), Levina et al. (1980), Mirzabekov et al. (1989)) (represented in figure 1) and is analogous to the Maxam and Gilbert DNA sequencing methodology (Maxam and Gilbert (1977)) as first proposed for adenine and guanine bases (Gilbert et al. (1976)).

The crosslinking reaction is initiated via methylation of the purine residues with dimethyl sulfate (Lawley and Brookes (1963)) (step 1). Guanine is methylated at N7 and adenine at N3 with the reactivity of guanine being approximately 10 times that of adenine (Mirzabekov et al. (1977)). Methylation destabilises the glycosidic bond of the purine (Lawley and Brookes (1963), Kriek and Emmelot (1964)) and allows bond scisssion at neutral pH with slightly elevated temperatures, usually 37°C or 45°C (step 2). Since the $t_{1/2}$ for cleavage of the glycosidic bond of methylated adenine is about 5 times less than that of methylated guanine at 37°C (Lawley and Brookes (1963)), the rate of depurination is roughly similar for the two purines. Depurination results in an aldehyde at C1 of the ribose which can then react with a nucleophilic amino acid side chain in close proximity (step 3). The formation of the Schiff base results in single-stranded scission of the DNA 3' to the point of attachment. This scission is the product of a β elimination reaction catalysed by the Schiff base (step 4). The Schiff base is acid labile (Cordes and Jenks (1963)) and therefore is usually reduced with $NaBH_4$ (step 5). Until recently it was postulated that this protocol would crosslink proteins to DNA mainly through their lysine residues. It became apparent, however, that histidine is the preferred nucleophile (Ebralidse et al. (1988), Mirzabekov et al. (1990)) and a revised protocol favouring lysine has been



see overleaf.

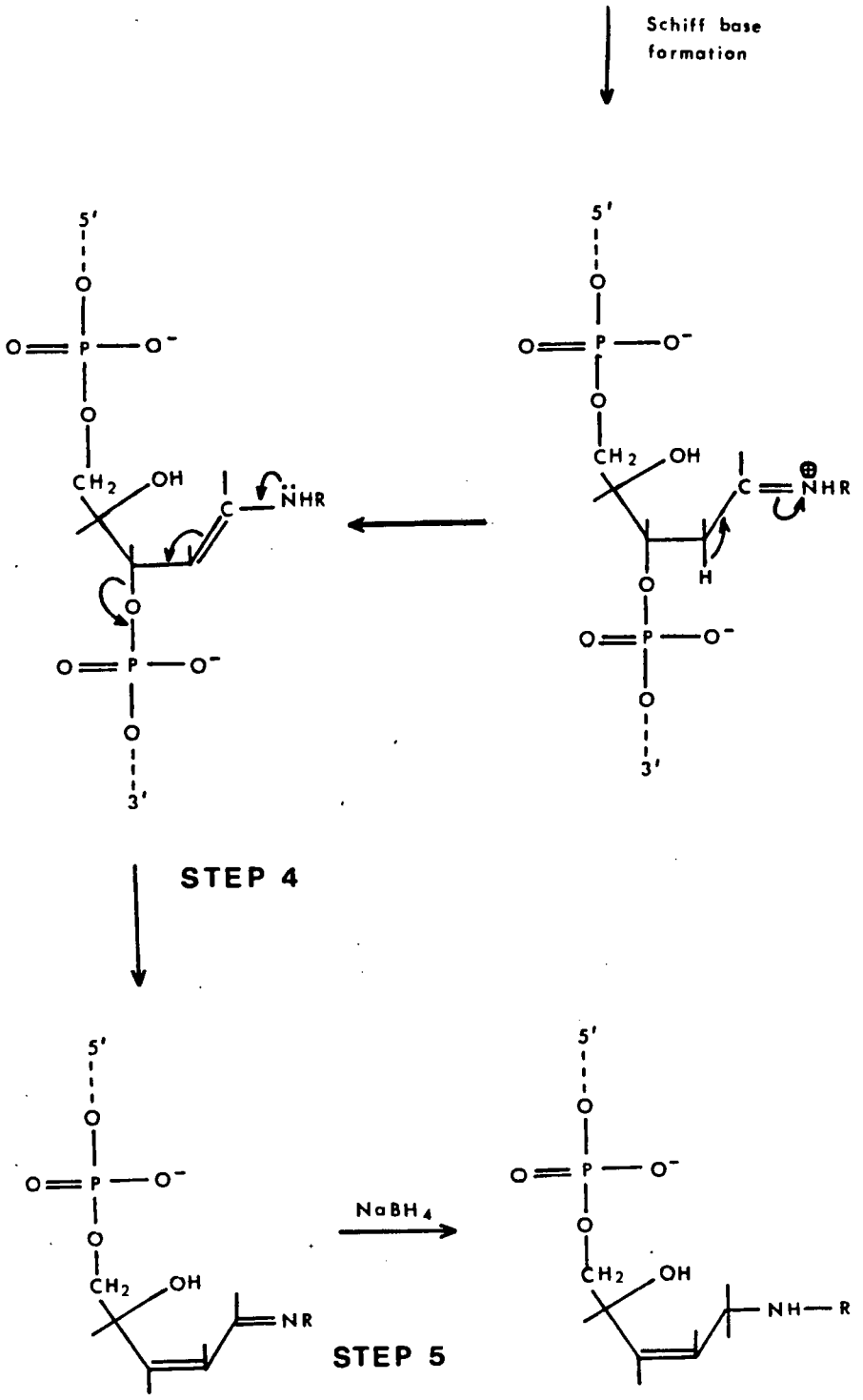


Figure 1.

Schematic diagram of the chemistry of covalent attachment of histone amines to single-stranded DNA.

devised (Nacheva et al. (1989)). This uses a milder reducing agent, sodium cyanoborohydride, which reacts preferentially with >C=N- rather than >C=O- (Mirzabekov et al. (1989)) and allows the reduction of the Schiff base to occur during steps 2, 3 and 4. Though the two protocols have been shown to favour histidine or lysine respectively (Nacheva et al. (1989)), the exact mechanisitic details have not yet been published. The experimental protocol for the histidine crosslinking methodology is presented in section 8.2 (see below).

If 5'-end-labelled core particles are crosslinked using the above methodology and then subjected to extensive DNA digestion, the histone(s) in closest proximity to 5'-end will prevent digestion of the 5'-end and hence will retain the label. The first attempt by Simpson (1976) indicated that H3 and H4 were near the 5'-ends of the DNA. This result however was shown to be compromised by the presence of an endogenous protein kinase and experiments that excluded this artifact showed that only H3 was weakly bound at this position (Simpson (1978b)).

A more sophisticated approach utilizing the scission of the DNA at the point of crossslinking was devised by Mirzabekov and co-workers (Mirzabekov et al. (1977)), Mirzabekov et al. (1978)). The positions of contact relative to the 5'-end are directly determined by the length of single-stranded DNA attached to each histone. It is important that the crosslinking level is kept below one histone per core particle as the binding of more than one histone to the same DNA strand would mask crosslinking sites with increasing distance from the 5'-end. A two dimensional gel system was used to determine the length of the DNA attached to the individual histones. Briefly, the crosslinked core particles were 5'-end-labelled with ^{32}P and denatured. After electrophoresis in a first dimension denaturing gel the crosslinked complexes would be separated due to both the histone component and the length of DNA attached. The histones were then hydrolysed in the first dimension gel strip before electrophoresis in a second

dimension denaturing gel. The DNA should then separate into discrete diagonals dependent on the mobility of the attached histone in the first dimension. All the diagonals cannot usually be resolved necessitating the parallel electrophoresis in the first dimension of denatured crosslinked core particles in which the histones have been labelled with ^{125}I. After hydrolysis of the DNA in this first dimension gel strip, the histones were electrophoresed in a second dimension denaturing gel. Spots on the same vertical line in both second dimension autoradiograms would originate from the same crosslinking site as the differently labelled crosslinked core particles were run on the same first dimension gel.

"The experiment is complicated" (van Holde (1989)) and the linear order of histone-DNA contacts (the primary organization of the core particle (Mirzabekov et al. (1978))) has been refined as the methodology has been perfected ((Mirzabekov et al. (1978), Shick et al. (1980), Mirzabekov et al. (1982), Bavykin et al. (1985)). The most comprehensive study to date was the comparison of the primary organisations of core particles from several species representing all three higher eukaryote kingdoms (Bavykin et al. (1985)). Very few differences were found between the various species, adding support to the postulate that the core nucleosome structure is highly conserved (Kornberg (1977)). The main histone-DNA contacts along one core DNA strand are: $5'H2B_{25,35}$-$H4_{55,65}$-$H3_{75,85,95}/H4_{88}$-$H2B_{105,115}$-$H2A_{118}$-$H3_{135,145}/H2A_{145}$ 3' (Bavykin et al. (1985)). If a dyad axis of symmetry is assumed (Klug et al. (1980)), then a three dimensional model can be obtained from the above contacts (Bavykin et al. (1985).

The contacts shown above were present in core particles that were crosslinked in nuclei before their isolation; core particles that were crosslinked after isolation had identical contacts except for an additional H2A contact at 75 bases from the 5'-end. This H2A contact was also absent from H1 containing nucleosomes (Belyavsky et al. (1980)). As detailed

above (section 1.4), the exact location of H2A in this area differs in the crystal structures of Uberbacher and Bunick (1989) and Richmond et al. (1984). It has been suggested that this could be caused by differences in the methods employed to obtain the respective electron density maps (Struck et al. (1992)) but it is possible that this could be a reflection of some type of H2A rearrangement.

It has been suggested that the several weaker histone-DNA contacts are due to oscillation of the histone residues from a favoured contact to a less favoured contact on the complementary strand (Belyavsky et al. (1980)) although they could also be explained by the presence of minor nucleosomal conformers (Bavykin et al. (1985)).

The model of the core particle derived from the histone-DNA contact sites correlates closely with data from other investigations of the core particle referred to above. The lack of crosslinking within the first 20 bases is in agreement with the lower thermostability of these regions (Weischet et al. (1978), Simpson (1979)). The argument that the shorter DNA fragments could be lost during precipitation has been directly addressed (Shick et al. (1980)) with no appearance of contacts in this area. Regions of little or weak contacts (40-50 bases and 122-130 bases from the 5'-end) are preferentially accessible to DNase 1 (Lutter (1978)). The contacts are roughly divided into a central region (occupied by H3 and H4) and a flanking regions (occupied by H2A and H2B). H2B is closest to the tetramer and it is also similarly situated in the high resolution diffraction study of the octamer (Albright et al. (1979)). H3 also contacts the 5'-end. It is also placed in this location by a similar crosslinking investigation (Simpson (1978b)) (see above) and by scanning transmission electron microscopy of platinum-labelled nucleosomes (Stoeckert et al. (1984)). The simultaneous involvement of H3 with the central and terminal regions is further support for the crucial role that the tetramer plays in stabilising the core particle structure (section 1.3.1).

The crosslinking methodology can also be used to identify the actual amino acid residue that is connected to the DNA (Ebralidse and Mirzabekov (1986), Ebralidse et al. (1988), Mirzabekov et al. (1990)). Very briefly, after crosslinking the DNA is digested away to a short tag which is then ^{32}P labelled. The amino acid/s which are associated with the ^{32}P label can then be identified using a variety of methodologies which have included mass spectometry and peptide map analysis. Though the concept is not complex and the logistics of the experiment are simplified by the preferential crosslinking of histidine, the procedure would appear to be very intricate. Only data for H5 (Mirzabekov et al. (1990)) and H4 (Ebralidse et al. (1988)) have been reported thus far. Histidine-18 of H4 is the main residue involved in the H4-DNA contacts. This histidine is embedded in a highly basic cluster which could be involved in the sharp bending of the DNA at sites ±1 (Richmond et al. (1984)) due to lateral asymmetric neutrilization of the DNA phosphates (Mirzabekov and Rich (1979), Manning et al. (1989)).

1.6 A core particle with two DNA supercoil turns

A chromatosome consisting of two full turns of DNA (166-168 bp), an octamer of core histones and histone H1 has been characterised (Simpson (1978a)). H1 is associated with the 20 extra base pairs, a symmetrical extension of 10 bp on both ends. H1 stabilizes this DNA at the entry and exit points; if H1 is removed from the chromatosome, however, the structure of the DNA wrapped around the core histone octamer, as measured by circular dichroism spectroscopy, remains unchanged (Simpson (1978a)). This suggests that the extra 20 bp of DNA might also interact with the core histones.

Todd and Garrard (1977), analysing miccrococcal nuclease digests of nuclei with a two dimensional electrophoretic technique, found a fraction (MII) containing only core histones and DNA of 160-185 bp. Protection of 168 bp of DNA

from microccocal nuclease digestion was found in nuclei depleted of H1, when digestions were carried out in 0.3-0.4 M NaCl (Weischet et al. (1979)). Tatchell and van Holde (1979) reconstituted core histones onto DNA fragments of defined lengths. Reconstitution onto a fragment of 161 bp gave a DNase 1 digest pattern identical to that of a 146 bp fragment; a 177 bp fragment, however, gave a smeared pattern. They suggested that the core histones are able to bind 165-170 bp of DNA and that the 177 bp fragment can therefore bind in a variety of ways.

A 167 bp core particle has been isolated from H1 stripped chicken erythrocyte chromatin and characterised (Lindsey and Thompson (1989)). The extra 20 bp are two extensions of 10 bp on both ends as for the chromatosome. Microccocal digestion of the 167 bp core particle proceded through a 156 bp intermediate. The central 100 bp of DNA are most tightly bound and evidence from circular dichroism spectroscopy and thermal denaturation suggests that the DNA winding and histone-DNA interactions increase as the terminal 20 bp are removed. Confirmation that the histone octamer can organise 2 full turns of DNA was obtained by Bavykin et al. (1990) who carefully analysed the MNase digestion products of crosslinked and non-crosslinked sea urchin sperm nuclei. They also demonstrated that the 146 bp core particle was obtained from the 2 turn core particle via an intermediate with only one 10 bp extension.

CHAPTER 2

HISTONE-DNA CONTACTS IN THE 167 BP CORE PARTICLE

2.1 Introduction

Physico-chemical characterisation of a core particle containing 167 bp or 2 full turns of DNA revealed that the extra 20 bp DNA of this particle result from 10 bp extensions at either end of the 146 bp core particle. Weaker histone-DNA interactions were found to occur in the 167 bp core particle compared with the 146 bp core particle (Lindsey and Thompson (1989)). Specific details of the interactions between the various histones and the DNA would allow a fuller understanding of the differences between the 167 bp and 146 bp core particles. Apart from diffraction studies, the only other methodology that yields very detailed information is the histone-DNA crosslinking protocol devised by Mirzabekov and co-workers (section 1.5).

This methodology has previously revealed differences in the histone:DNA interactions between the chromatosome (Belyavsky et al. (1980)) and the 146 bp core particle (Bavykin et al. (1985)). Thus histone H3 was found to bind past the 146 bp limit in the chromatosome. In addition, the histone H2A contact close to the dyad axis found in isolated core particles was absent in the chromatosome. This contact was also absent in core particles that were isolated after crosslinking had taken place in the nucleus. Examination of the histone-DNA contacts in the 167 bp core particle should reveal whether this particle more closely resmbles the 146 bp core particle or the chromatosome.

2.2 Results and Discussion

2.2.1 Isolation and characterization of 146 and 167 bp core particles

146 bp and 167 bp core particles were isolated as described (Drew and Calladine (1987)) from chicken erythrocyte nuclei (section 8.1). This protocol has been found to be both reproducible and convenient. The core particles produced contained DNA of an homogeneous length and no linker histones (figure 2). Both these facets of the core particles were crucial for the investigation of their primary organisation. The core particles were found to contain DNA of lengths 146 ± 1 bp and 167 ± 2 bp respectively and a normal complement of the core histones.

2.2.2 The primary organisation of the 146 bp core particle

The crosslinking methodology and concomitant electrophoresis (section 8.2) was initially attempted on the 146 bp core particle to allow comparison with the published primary organisation of chicken erythrocyte core particles (Bavykin et al. (1985)).

2.2.2.1 Determination of crosslinking efficiency

The pattern of spots obtained in the two dimensional protein gels would be greatly complicated if more than one histone was crosslinked per core particle. The binding of more than one histone to the same DNA strand with the resultant cleavages, would proportionately mask crosslinking sites with increasing distance from the 5'-end. A crosslinking efficiency of 6.25 % of the histones in a core particle would give approximately one crosslink per two core particles. The efficiency of crosslinking was determined by comparing the protein:DNA ratio (section 8.2.1.2) in cetyltrimethyl ammonium bromide stripped

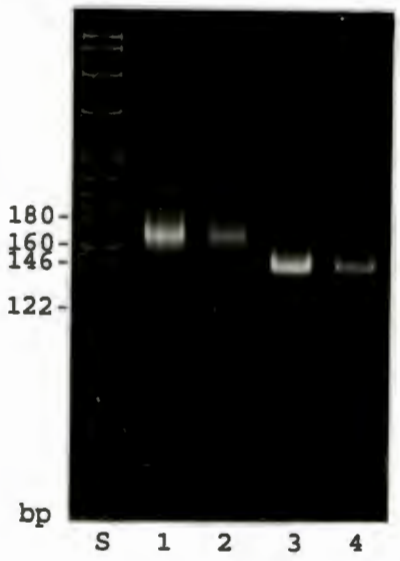


Figure 2.

Analysis of the DNA and histone content of 146 bp and 167 bp core particles.

a. 6 % non-denaturing gel of 167 bp (lanes 1 and 2) and 146 bp (lanes 3 and 4) core particle DNA. Two loadings of each preparation are shown, that in lanes 2 and 4 being 3 times of that in lanes 1 and 3. The standard (S) is a *Hpa* II digest of pBR 322.

b. SDS PAGE of the histones associated with the 167 bp core particle. The standard (S) is a total acid extract of chicken erythrocyte nuclei.

(section 8.2.1.1) crosslinked core particles to that in crosslinked core particles. The background protein:DNA ratio was determined using cetyltrimethyl ammonium bromide stripped normal core particles. The efficiency of the crosslinking was found to be 7.5 (±3) %. This value is close to that of approximately 5 % reported in (Mirzabekov et al. (1989), Shick et al. (1980)) and below that of 12.5 % required for crosslinking one histone per core particle. The possibility of a second crosslink in the same core particle was between 0.6 % (for 7.5 % crosslinking) and 1.6 % (for 12.5 % crosslinking).

2.2.2.2 Depletion of uncrosslinked histones and DNA

The low level of crosslinking used in determining the primary organisation of the core particles requires exhaustive depletion of free (uncrosslinked) histones and DNA. The cetyltrimethyl ammonium bromide stripping procedure routinely removed 95 (±3) % of the proteins in a normal core particle. It has been suggested that the 5 % of the proteins that could not be stripped off are tightly bound non histone proteins (Levina et al. (1981)). The use of hydroxyapatite chromatography in SDS to remove free DNA (section 8.2.2.3) exploits the binding of SDS-protein complexes to hydroxyapatite. Single-stranded DNA elutes at 150 mM NaH_2PO_4 and SDS-protein-DNA complexes at 500 mM NaH_2PO_4. 85-90 % of ^{32}P endlabelled heat denatured core particle DNA eluted at 150 mM NaH_2PO_4. In addition when ^{125}I labelled crosslinked core particles were chromatographed, iodinated material only eluted at 500 mM NaH_2PO_4.

2.2.2.3 Two dimensional 'DNA' electrophoresis

As detailed in (sections 1.5 and 8.2.3) the two dimensional DNA gel system involved electrophoresis of a ^{32}P end-labelled crosslinked complex in the first dimension followed by removal of crosslinked histones by proteolysis during electrophoresis in the second dimension. The different mobilities of the attached core histones resulted in diagonals of labelled DNA fragments corresponding to each histone.

The ^{32}P endlabelled crosslinked 146 bp core particles were electrophoresed in a 17 % acrylamide, 0.085 % methylenediacrylamide first dimension gel and 15 % acrylamide, 0.075 % methylenediacrylamide second dimension gel. The autoradiogram is shown in figure 3 and a schematic thereof in figure 4. A comparison of the contacts obtained here with those described previously (Bavykin et al. (1985)) for the 146 bp core particle (a compilation of data from chicken erythrocyte, *Drosophila* embryo and yeast) is shown in Table 1.

Table 1.
Comparison of histone-DNA contact sites derived from figure 2 (146) with those described by Bavykin et al. (1985) (146_p)

Histone	146_p	146	Histone	146_p	146
H3	35*		H4	45	43
	48*			55	54
	58*			65	64
	68	68		77	76
	75	78		90	88
	85	86		97	95
	95	95			
		122			
	135	135			
	142	142			
H2B	25		H2A	35*	
	30*			75	74
	35	36		115	115
	40	41		135	135
	50	52		142	141
	85	86			
	95	99			
	105	108			
	115	115			
	125	124			

Though the diagonals for histones H2A and H2B were not resolved, the spots obtained on the H2A/H2B diagonal were a combination of the crosslinking sites for these two histones (Bavykin et al. (1985)) and were therefore assigned accordingly. Some very low intensity spots (marked by asterisks in the above table) observed by Bavykin et al. (1985) could not be detected. A further crosslinking site for

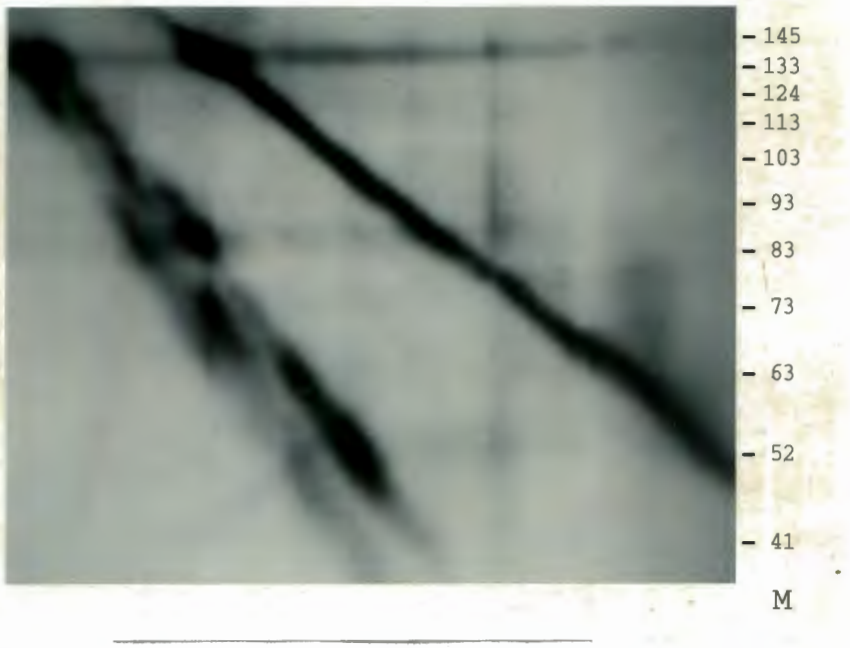


Figure 3.

Autoradiogram of two-dimensional 7 M urea SDS PAGE of ^{32}P end-labelled single-stranded DNA from crosslinked 146 bp core particles. Electrophoresis of crosslinked histone-DNA complexes in the first dimension (from left to right) was carried out in a 17 % polyacrylamide gel 250 x 250 x 0.5 mm. After pronase digestion to degrade the crosslinked histones, the second dimension electrophoresis was carried out in a 15 % polyacrylamide gel 250 x 250 x 1 mm. A DNase 1 digest of chicken erythrocyte nuclei (Lohr and van Holde (1979), Prunell et al. (1979)) was co-electrophoresed in order to size the labelled single-stranded frangments; the gel was stained with ethidium bromide and the positions of the bands from the DNase 1 digest marked. The dashes on the right hand side of the gel (marked M) denote the position and length in bases of each band.

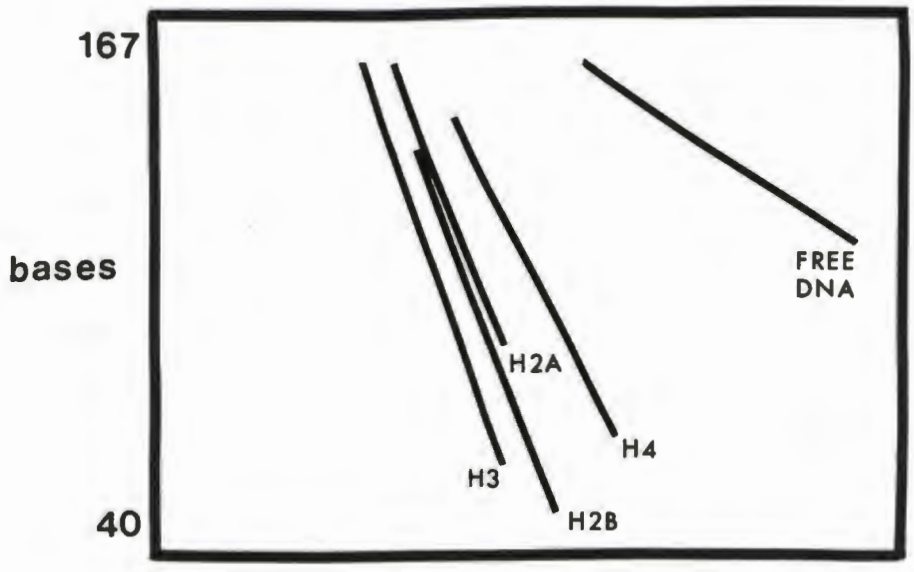


Fig 4.

Schematic representation showing the relative positions of the diagonals originating from free DNA and from DNA crosslinked to histones H3, H2B, H2A, H4 in the two-dimensional gel system. This schematic is representative of the 167 bp core particle; the diagonals of histones H2A and H2B were not resolved in the case of the 146 bp core particle.

histone H2B at 25 bases from the 5'-end has probably been electrophoresed off the bottom of the second dimension gel. An additional histone H3 crosslinking site at 122 bases was observed. This is, however, close to an area of tight histone H3 binding. The pattern obtained for the 146 bp core particle was essentially very similar to those published previously with all the major areas of binding for the different histones being identical. Histone H3 occupies the central and 5'-end regions, histone H4 the central region and histones H2A and H2B the regions flanking the center. This data was convincing evidence that the two dimensional DNA electrophoresis system could be utilized for investigation of the primary organisation of the 167 bp core particle.

2.2.2.4 Two dimensional 'protein' electrophoresis

If all the histone diagonals were not resolved in the two dimensional DNA gel system, then a two dimensional protein gel system could be used to attempt to identify from which histone the DNA spots originated. The detailed description of this electrophoretic system is given in (sections 1.5 and 8.2.3.3). Briefly, after electrophoresis of the ^{125}I labelled crosslinked complexes in the first dimension and before electrophoresis in the second dimension, the crosslinked DNA was hydrolysed with formic acid/ diphenylamine. If the ^{32}P and ^{125}I labelled complexes were run on the same first dimension gel, spots on the same vertical line in both second dimension autoradiograms would originate from the same crosslinking site. The ^{125}I labelled crosslinked core particles were electrophoresed in a 17 % acrylamide 0.085 % methylenediacrylamide first dimension gel and a 20 % acrylamide 0.1 % methylenediacrylamide second dimension gel. Autoradiograms of the gel with the highest achieved resolution and a typical gel are shown in figure 5. Some spots, which corresponded to crosslinking sites that had been determined in the two dimensional DNA gels, could be discerned (see figure 5a). These mainly originated from H4 crosslinks as this

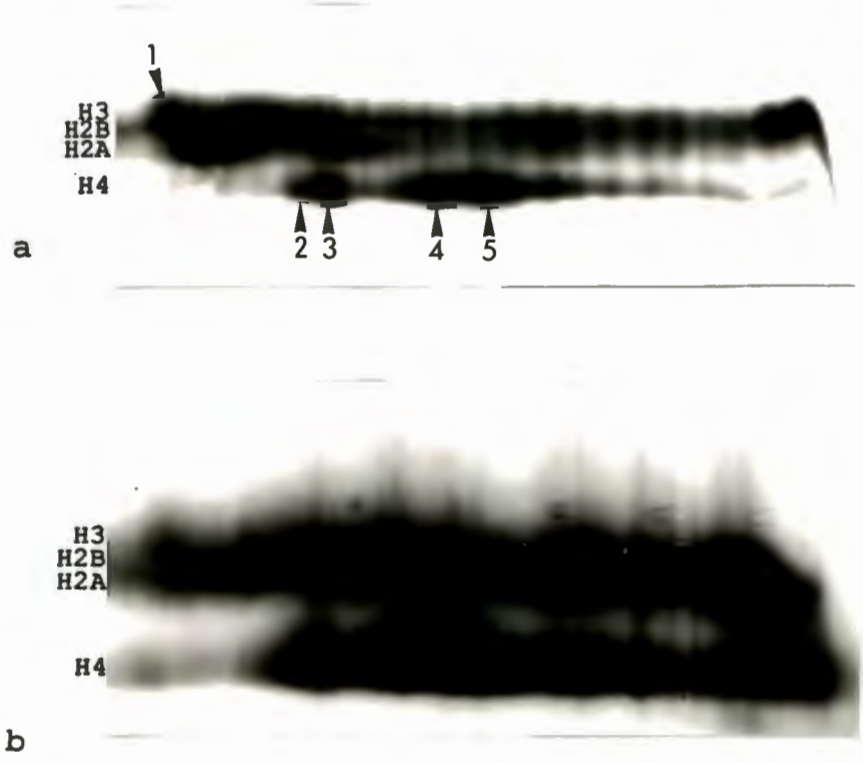


Figure 5.

Autoradiograms of two-dimensional SDS PAGE of ^{125}I labelled histones from crosslinked 146 bp core particles. Electrophoresis of crosslinked histone-DNA complexes in the first dimension (from left to right) was carried out in a 17 % polyacrylamide gel 250 x 250 x 0.5 mm. After hydrolysis of the attached DNA by formic acid/diphenylamine treatment, second dimension SDS PAGE was carried out in a 20 % polyacrylamide gel 400 X 350 X 1 mm.

a. Highest resolution autoradiogram achieved. The arrows indicate resolved spots tentatively assigned as: 1:H3 at 142 bases, 2:H4 at 95 bases 3:H4 at 90 bases 4:H4 at 65 bases 5:H4 at 54 bases.

b. Autoradiogram illustrating the usual streaking and resolution observed.

histone was well resolved. No additional data, however, could be gained from these gels for two reasons.

These were :

(1) The spots were obscured by vertical streaking which were more intense in the region of the histone spots. The overall intensity of the streaking varied from gel to gel but was always visible.
(2) The histones H2A and H2B spots were once again unresolved.

As it was not clear what the reason or reasons were, several control experiments were carried out. The efficiency of the DNA hydrolysis was determined by running ^{32}P end-labelled crosslinked core particles through the two dimensional protein electrophoretic system. Figure 6 shows that formic acid treatment resulted in complete disappearance of the ^{32}P labelled DNA.

It could be argued that this did not show complete removal of DNA but merely removal of the radioactive label. Therefore the experiment was repeated using a silver staining protocol (Oakley et al. (1980)) with 100 μg 146 bp core particle DNA. Silver staining could detect 0.05 μg in a 1 cm wide band. No DNA was visible in the formic acid treated gel (figure 7).

Both results indicated that DNA hydrolysis in the gel strip was complete. The electrophoretic resolution of histones in the gel system was therefore examined. If iodinated chicken erythrocyte core histones were taken through the entire two dimensional electrophoretic process (including formic acid/ diphenylamine treatment) H3 and H2B were not resolved (figure 8). The acrylamide concentration was increased to 22.5 % in the first and second dimension gels and this resulted in complete resolution of all the histones (figure 9).

Figure 6.

Determination of the efficiency of DNA degradation by formic acid/diphenylamine hydrolysis.

I: radioactivity determination

^{32}P end-labelled non-crosslinked 146 bp core particles were heat denatured and electrophoresed in a 7M urea 0.1 % SDS first dimension gel. The gel strip was formic acid/diphenylamine treated before electrophoresis in the second dimension. Autoradiography of this gel failed to detect any radioactivity. An identical sample was subjected to the same protocol except that the formic acid/diphenylamine treatment was omitted. The autoradiogram is shown; the arrow indicates the position of 146 bases of DNA.

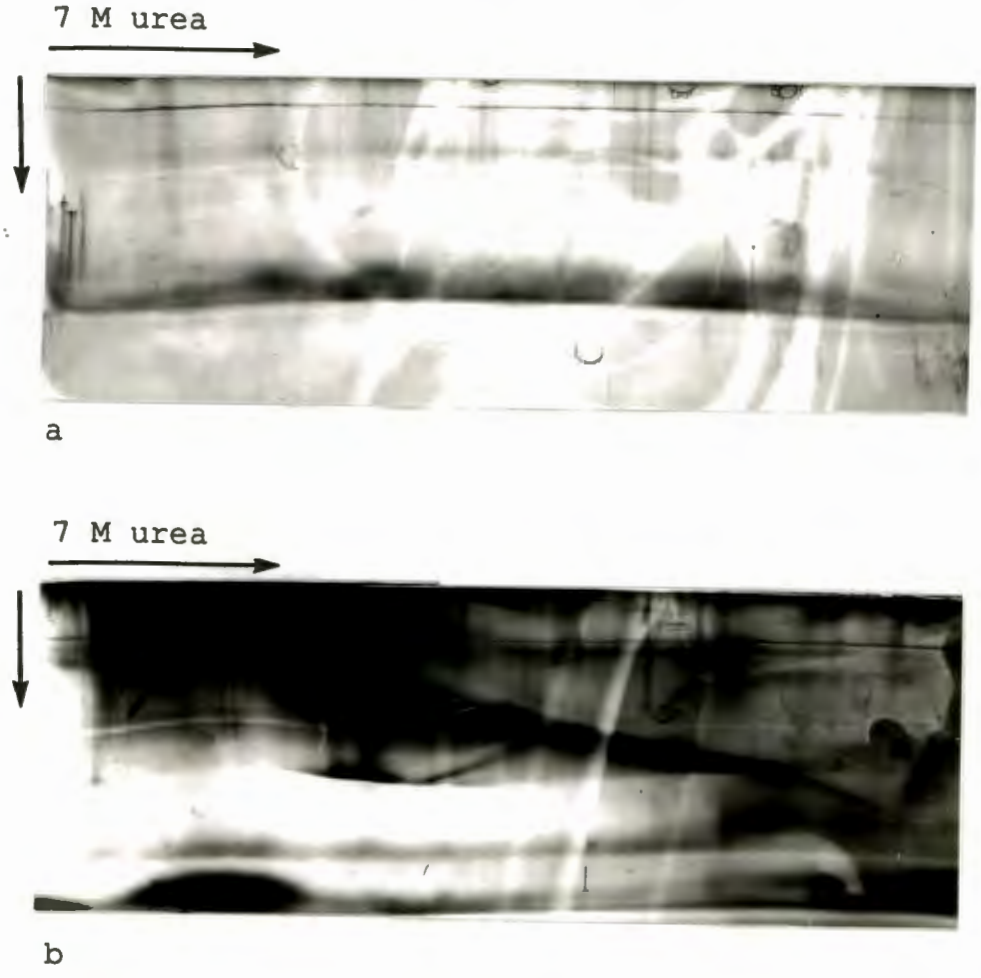


Figure 7.

Determination of the efficiency of DNA degradation by formic acid/diphenylamine hydrolysis.

II: silver staining.

a. Two-dimensional SDS PAGE of 100 μg single-stranded DNA from 146 bp core particles. The protocol was as for figure 6 except that the DNA was unlabelled and the gel subjected to silver staining.

b. As above except the first dimension gel strip was not subjected to formic acid/diphenylamine treatment. The arrow indicates the position of 146 bases DNA.

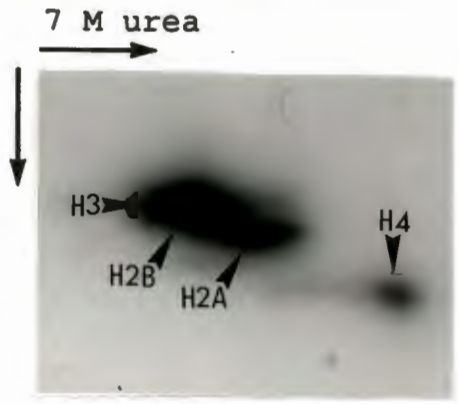


Figure 8.

Determination of the resolution of the formic acid/diphenylamine two-dimensional protein gel system.

Autoradiogram of two-dimensional SDS PAGE of ^{125}I labelled core histones. After heat denaturation and electrophoresis in a 7M urea 0.1 % SDS first dimension gel (resolving gel: 17 % acrylamide 0.085 % methylenediacrylamide), the first dimension gel strip was formic acid/diphenylamine treated and then electrophoresed into a SDS second dimension gel (resolving gel: 20 % acrylamide 0.1 % methylenediacrylamide).

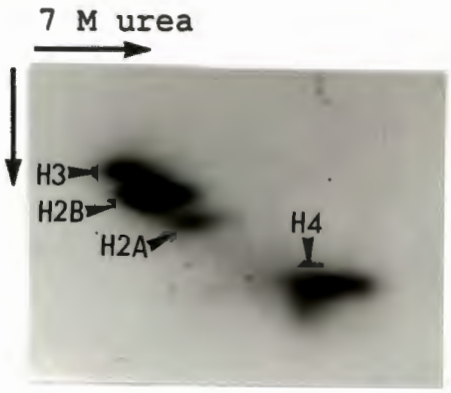


Figure 9.

Determination of the resolution of the formic acid/diphenylamine two-dimensional protein gel system with increased polyacrylamide concentration.

Autoradiogram of two-dimensional SDS PAGE of ^{125}I labelled core histones. After heat denaturation and electrophoresis in a 7M urea 0.1 % SDS first dimension gel (resolving gel: 22.5 % acrylamide 0.1125 % methylenediacrylamide), the first dimension gel strip was formic acid/diphenylamine treated and then electrophoresed into a SDS second dimension gel (resolving gel: 22.5 % acrylamide 0.1125 % methylenediacrylamide).

These conditions, however, had no effect on the resolution when crosslinked core particles were used as the substrate.

I then corresponded with Prof. K.K. Ebralidse, one of the Russian workers involved in establishing the crosslinking methodology. He suggested that either a slow dissociation of soluble histone aggregates or oxidative and/or highly adsorptive gels might be responsible for the above problems. Accordingly, the following attempts were made to address the former suggestion

(a) The number of ethanol precipitations was minimized.

(b) 7 M urea was introduced in the 0.1 % SDS 0.125 M Tris-HCl pH 6.8 buffer for washing prior to electrophoresis, the second dimension stacking gel and the gel used to polymerise the first dimension strip.

(c) The crosslinked complex in sample application buffer (section 8.2.2.1) was centrifuged at 15000 g for 5 minutes to remove any dust prior to electrophoresis.

The following attempts to address the latter suggestion:

(a) The highest quality gel reagents were used (section 8.2.4).

(b) The gel solutions were treated with charcoal and a mixed cation/anion exchange resin (section 8.2.4).

(c) Thioglycollic acid (a negatively charged reducing agent) was introduced in the upper reservoir chamber for both the first and second dimension.

These modifications unfortunately resulted in no discernible improvement in resolution of spots or diminution of streaking.

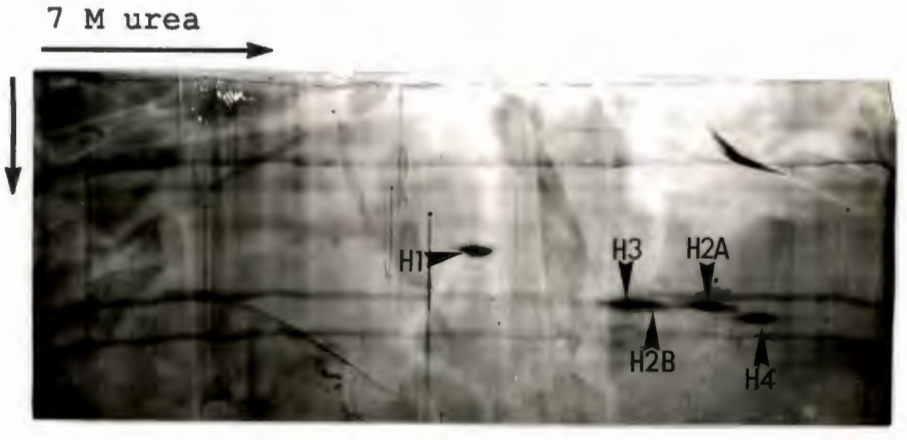
2.2.2.4.1 Nuclease S1 hydrolysis of crosslinked DNA

It was thought that an alternative, less harsh, method of removing the DNA might solve the above problems. An enzymatic approach, similar to that employed for the DNA gel system, was appealing because any chemical modifications to the histones

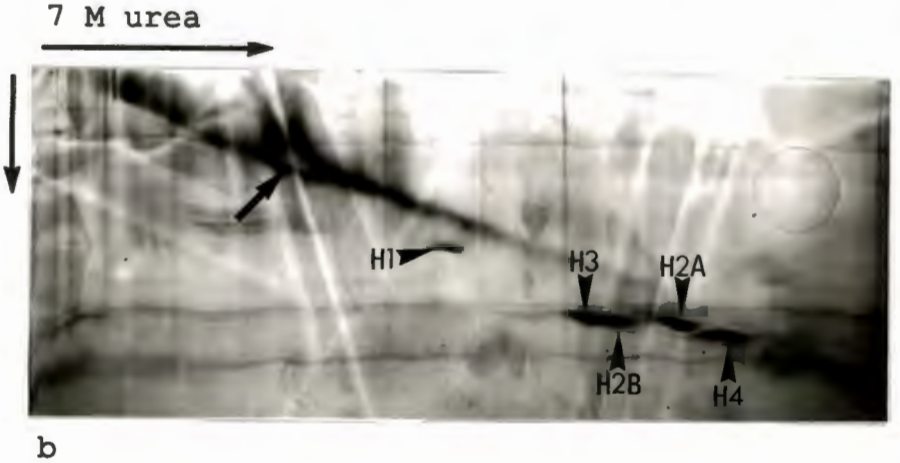
would be minimized. Nuclease S1 from *Aspergillus oryzae* was considered a suitable enzyme. This single-stranded nuclease retains 50 % activity in 0.1 % SDS (w/v) (Vogt (1973)) and is active in high concentrations of urea (Zechel and Weber (1977)). A gel system was devised which incorporated Zn++ and a pH of 4.3, both of which are requirements for nuclease S1 activity (section 8.2.3.3.2). Using this gel system, no DNA could be detected by silver staining after digestion of 25 μg of denatured 146 bp core particle DNA with 400 units (section 8.2.3.3.2) of nuclease S1 (figure 10). When iodinated chicken erythrocyte core histones were taken through this gel system (using 4000 units of nuclease S1) sharp spots with no suggestion of proteolysis were obtained (figure 11).

This system was, therefore, applied to the crosslinked core particles. The results were similar to those achieved by formic acid treatment. The intensity of the streaking again varied considerably from gel to gel. The best result is shown in figure 12. There appeared to be resolution of histones H2A and H2B in this particular gel. The data unfortunately could not be used for two reasons:
1. the presence of two additional rows of spots just above and below the H4 spots.
2. the quality of this gel was not reproducible even after numerous attempts.

Use of the two dimensional protein gel system to elucidate the histone H2A and H2B spots has proved elusive. Reason(s) for the irreproducibility of the protein gel and the vertical streaking even when using the same crosslinked core particle preparation is/are unknown. The good resolution of the DNA gels argues against protein damage during the crosslinking procedure. The sharp spots attained with iodinated core histones would indicate that the iodination procedure is not a problem either. The problem/s must arise either during or after the treatment of the first dimensional strip. Core histones do not seem to be adversely effected by formic acid or nuclease S1 treatment. Absolute removal of DNA from the



a



b

Figure 10.

Determination of efficiency of DNA degradation by S1 nuclease hydrolysis.

a. Two-dimensional SDS PAGE of 25 μg single-stranded DNA from 146 bp core particles together with 10 μg total acid extracted chicken erythrocyte histones. After heat denaturation and electrophoresis in a 7M urea 0.1 % SDS first dimension gel, the first dimension gel strip was polymerised on top of a 1 mm thick 0.1 % SDS gel containing a 25 mm deep stacking gel. Electrophoresis was carried out with an overlay of 400 units S1 nuclease until the bromophenol blue dye front had migrated 15 mm into the stacking gel. The stacking gel was excised and incubated in 100 volumes 50 mM sodium acetate 0.1 M NaCl 1 mM $ZnSO_4$ pH 4.3 at 37°C with 5 buffer changes at 15 minute intervals and a final incubation period of 40 minutes. The stacking gel was then washed into 0.1 % SDS 0.125 M Tris-HCl pH 6.8. After this series of treatments, the stacking gel was finally electrophoresed on a "third" dimension 22.5 % SDS gel 1.5 mm thick which was subjected to silver staining.

b. As above except S1 nuclease was omitted. The arrow indicates the position of 146 bases DNA.

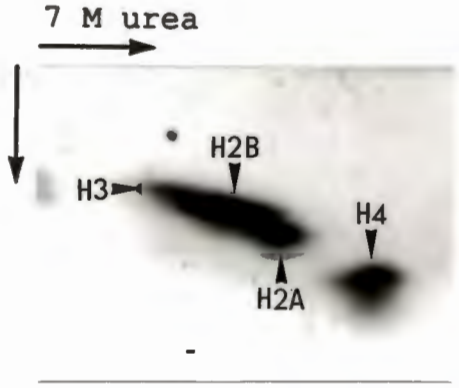


Figure 11.

Determination of the resolution of the S1 nuclease two-dimensional protein gel system.

Autoradiogram of two-dimensional SDS PAGE of ^{125}I labelled core histones. After heat denaturation and electrophoresis in a 7M urea 0.1 % SDS first dimension gel, the first dimension gel strip was treated as in figure 10 but with 4000 units S1 nuclease.

Figure 12.

Autoradiogram of two-dimensional SDS PAGE of ^{125}I labelled histones from the crosslinked 146 bp core particle.

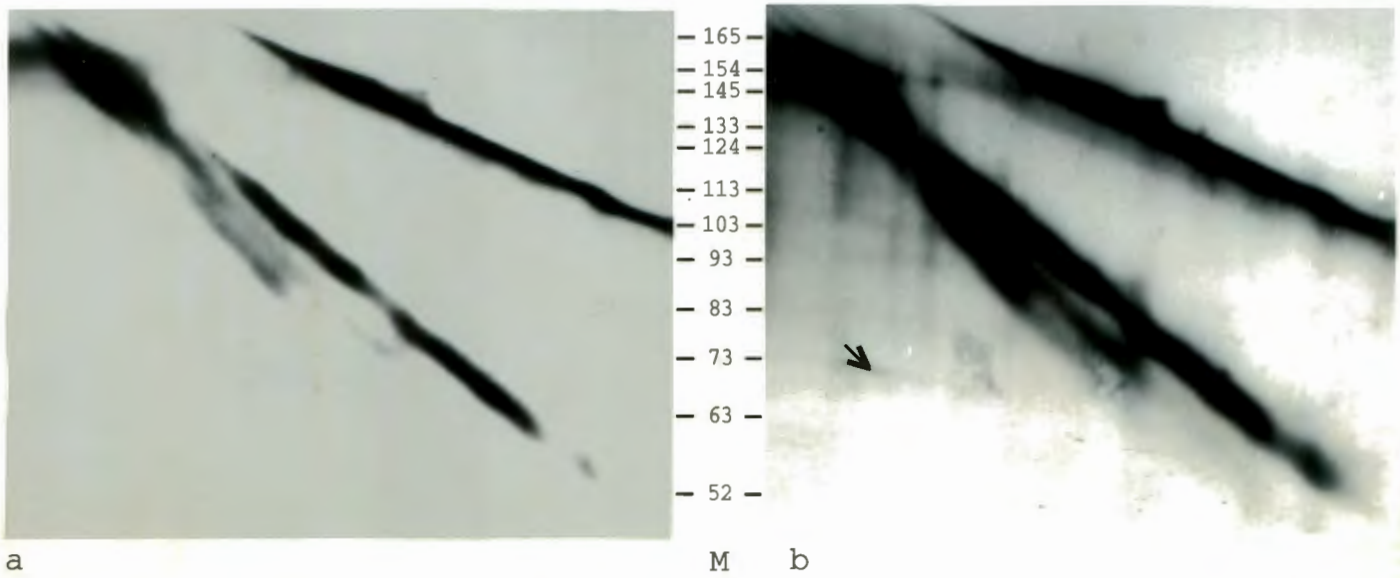
Electrophoresis of crosslinked histone-DNA complexes in the first dimension (from left to right) was carried out in a 22.5 % polyacrylamide gel 250 x 250 x 0.5 mm. After digestion of crosslinked DNA with 4000 units S1 nuclease (section 8.2.3.3.2), "third" dimension SDS PAGE was carried out in a 22.5 % polyacrylamide gel 400 X 350 X 1.5 mm. The histones are tentatively assigned as shown. The anomalous bands are indicated by arrows.

crosslinked histones has not been proved for either technique but increasing the duration of hydrolysis had no effect. A portion of the DNA could be inaccessible to hydrolysis but there is no obvious reason for this, particularly with the formic acid treatment.

2.2.3 The primary organisation of the 167 bp core particle

The acrylamide concentration in the first dimension DNA gel was increased to 22.5 % for the crosslinked 167 bp core particle. The higher concentration had been shown to enhance resolution of core histones (section 2.2.2.4). It was postulated that this might allow resolution of the histone H2A and H2B spots in the second dimension. Three different concentrations of acrylamide 12 %, 15 % and 18 % were used in the second dimension gel to allow resolution of spots of long, medium and short DNA length respectively. A synthetic single-stranded polydeoxyribonucleotide comprising 68 bases was introduced as a standard to facilitate sizing of the DNA spots. The autoradiograms are shown in figure 13. Close examination of different exposures of the autoradiograms of these gels showed that some spots on the H2A/H2B diagonal were shifted towards the H4 diagonal relative to the rest of the spots in the H2A/H2B diagonal. These would originate from H2A. The study of different levels of exposure also allowed resolution of adjacent spots in the various diagonals. The diagonals were also densitometrically scanned as an additional means of identifying these adjacent spots (the H2A/H2B diagonal was scanned as one diagonal) (figure 14).

The contact sites are shown in Table 2 and compared with those of the 146 bp core particle. No further contacts below 35 bases could be found even by increasing the acrylamide concentration in the two dimensional gel system to 25 % (data not shown).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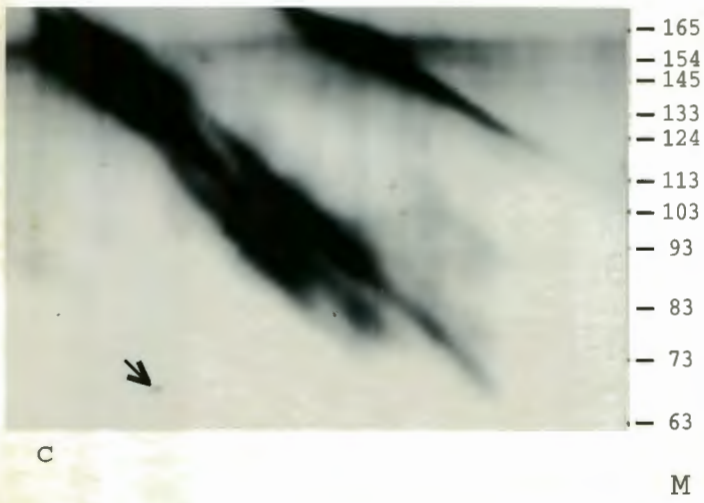
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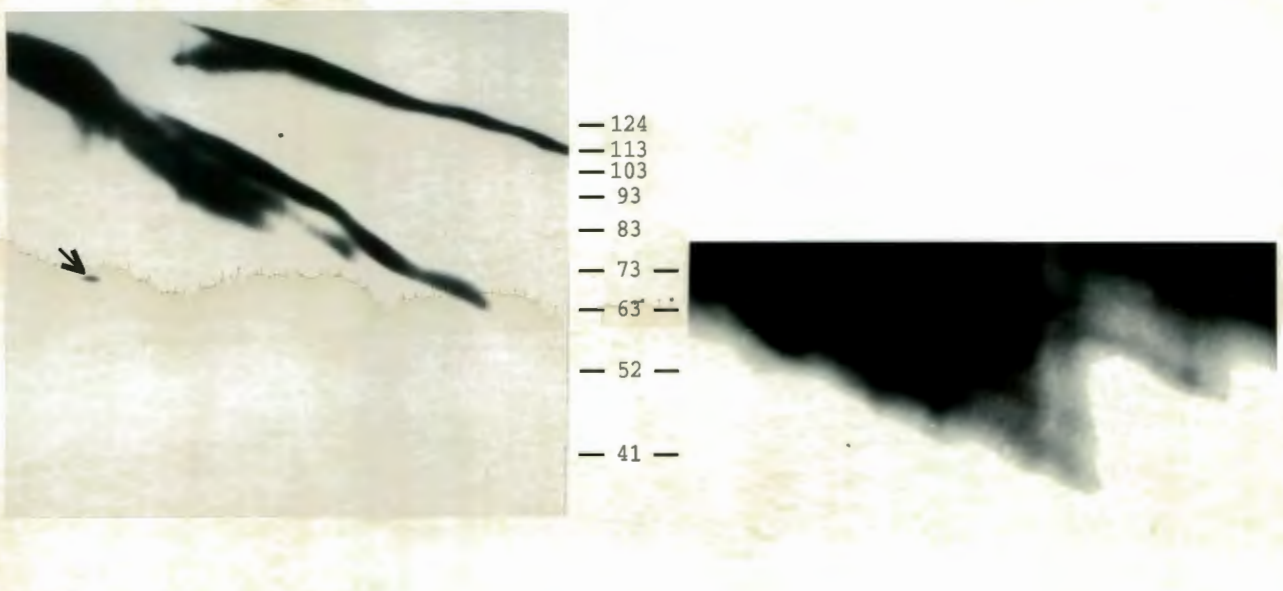
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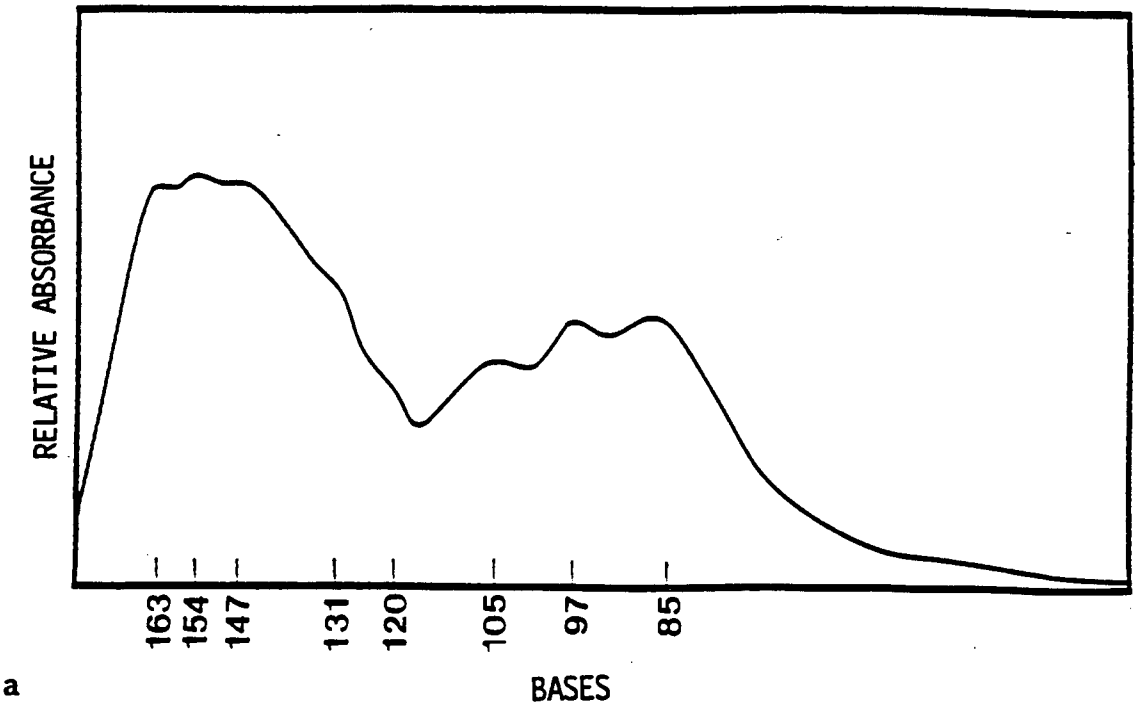
Figure 13.

Autoradiograms of two-dimensional 7 M urea SDS PAGE of ^{32}P end-labelled single-stranded DNA from crosslinked 167 bp core particles. Electrophoresis of crosslinked histone-DNA complexes in the first dimension (from left to right) was carried out in a 22.5 % polyacrylamide gel 250 x 250 x 0.5 mm. After pronase digestion, the second dimension electrophoresis was carried out in a polyacrylamide gel 250 x 250 x 1 mm. "A" and "b" are two exposures of a 15 % second dimension gel; "c" and "d" are 18 % and 12 % second dimensional gels respectively. "E" is a longer exposure of the bottom part of the autoradiogram shown in "d". The arrow indicates the position of the end-labelled synthetic 68 base polydeoxyribonucleotide.


RELATIVE ABSORBANCE
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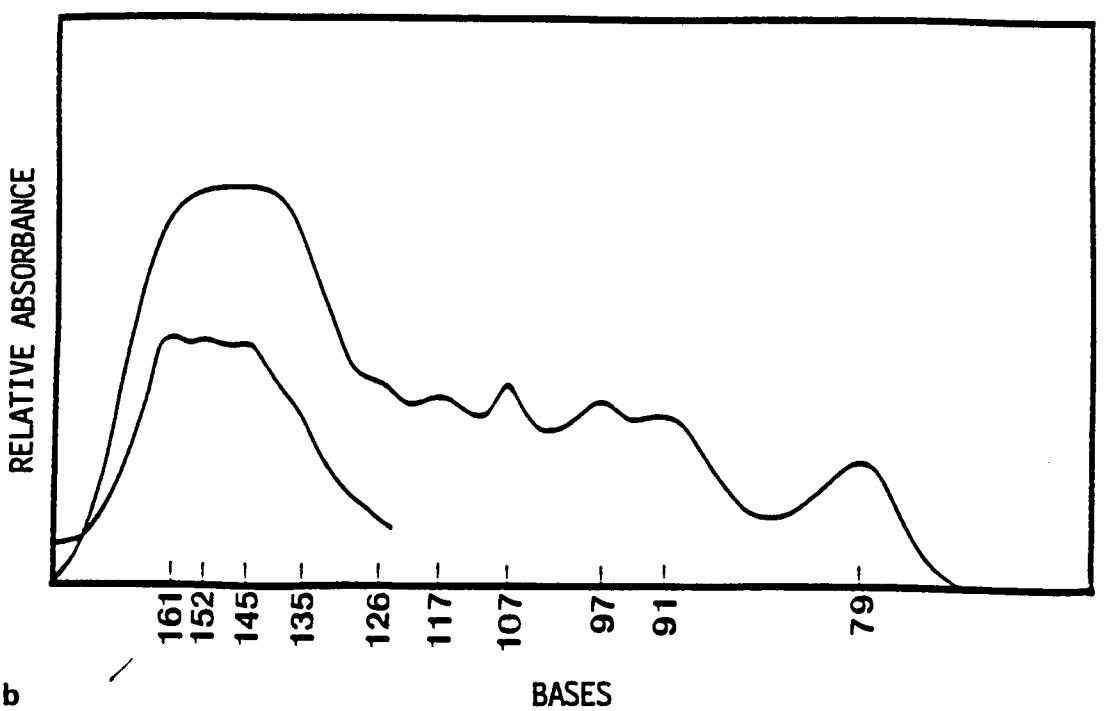
BASES

a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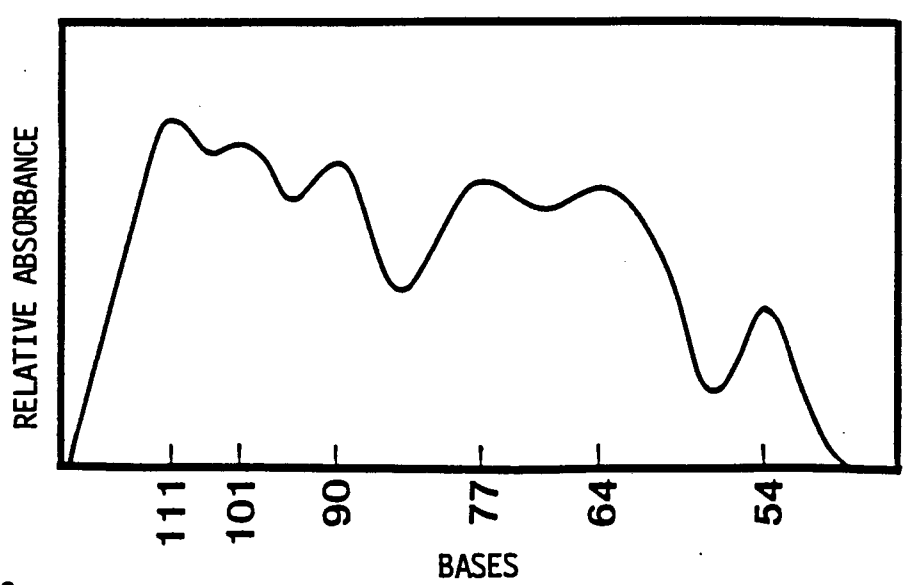

RELATIVE ABSORBANCE
111
101
90
77
64
54
BASES

Figure 14.

Densitometric scans of the diagonals originating from crosslinked histones of the gels shown in figure 13. a: histone H3; b: histone H2A/H2B; c: histone H4. The inset in "b" is a densitometric scan of the same diagonal from a shorter exposure.

Table 2.
Comparison of histone-DNA contact sites in the 167 bp core particle (167) with the contacts in the 146 bp described above (see Table 1) (146) and with those described by Bavykin et al. (1985) (146_p)

Histone	146_p	146	167	Histone	146_p	146	167
H3	35			H4			45
	48		58				47
	58				45	43	54
	68	68			55	54	64
	75	78	87		65	64	77
	85	86	97		77	76	90
	95	95	105		90	88	101
			120		97	95	111
		122	131				
	135	135	147				
	142	142	154				
			163				
H2B	25			H2A	35		
	30		40		75	74	79
			41		115	115	
	35	36	49		135	135	145
	40	41	55		142	141	152
	50	52	58				161
			91				
	85	86	97				
	95	99	107				
	105	108	117				
	115	115	126				
	125	124	135				

An estimation of the relative intensities of the 167 bp core particle contact sites is given in figure 15. The 167 bp core particle has symmetrical extensions of 10 bp of DNA at the entry and exit points (Lindsey and Thompson (1989)) relative to the 146 bp core particle. Therefore similar contact sites (defined as when the histone was bound to the same helical repeat relative to the dyad axis of the relevant core particle) in the two types of core particle would be elongated by 10 bases in the 167 bp core particle. Most of the 167 bp contact sites were elongated by 10 (±3) bases and consequently

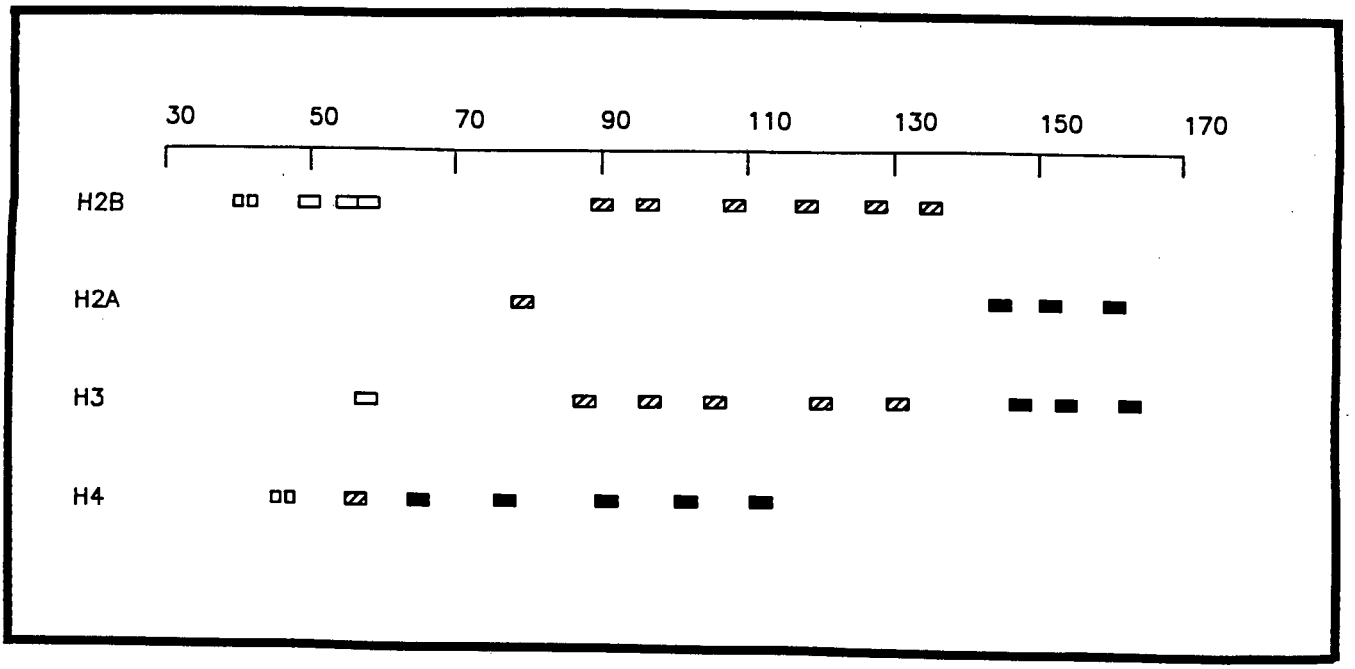


Figure 15.

Estimation of the relative intensities of all the histone-DNA contacts in the 167 bp core particle. The intensities have been graded as: high: filled boxes; intermediate: hatched boxes; low: open boxes.

fitted into this category. There were, however, several differences and these fell into distinct groups.

(a) Low intensity spots present in the 167 bp core particle that were not present in the data obtained for the 146 bp core particle. These contact sites could be found in the primary stucture of chicken erythrocyte core particles given by Bavykin et al. (1985). This was not surprising as the 167 bp core particle was studied in much greater detail. Sites in this group were histone H3 at 58 bases and histone H2B at 40/41 bases.

(b) Low intensity spots present in 167 bp core particles that were not present in the published 146 bp core particle (Bavykin et al. (1985)) and vice versa. Such spots were histone H3 at 68 bases, histone H2A at 115 bases in the 146 bp core particle and histone H4 at 45/47 bases in the 167 bp core particle. These might reflect minor differences in the primary organisation of the 167 bp and the 146 bp core particles.

(c) Spots that were present in both core particles but differed in intensity. The histone H4 spot at 90 bases in the 167 bp core particle was significantly more intense than the histone H4 spot at 76 bp in the 146 bp core particle. In addition, the intensity of the histone H2A spot at the dyad axis was less intense in the 167 bp core particle. The intensity of this spot was densitometrically compared with that of the histone H4 spot at approximately position 1.5 (figure 16); the histone H4 spot at this position was chosen as a standard because it was of similar intensity in both core particles. The intensity of the histone H2A spot was found to be reduced by approximately one half in the 167 bp core particle. The relevance of these changes is discussed in relation to those given in (e) (see below).

(d) Moderate intensity spots that were present only in the 167 bp core particle. These were a histone H3 spot at 120 bases and a histone H2B spot at 91 bases. These additional contact

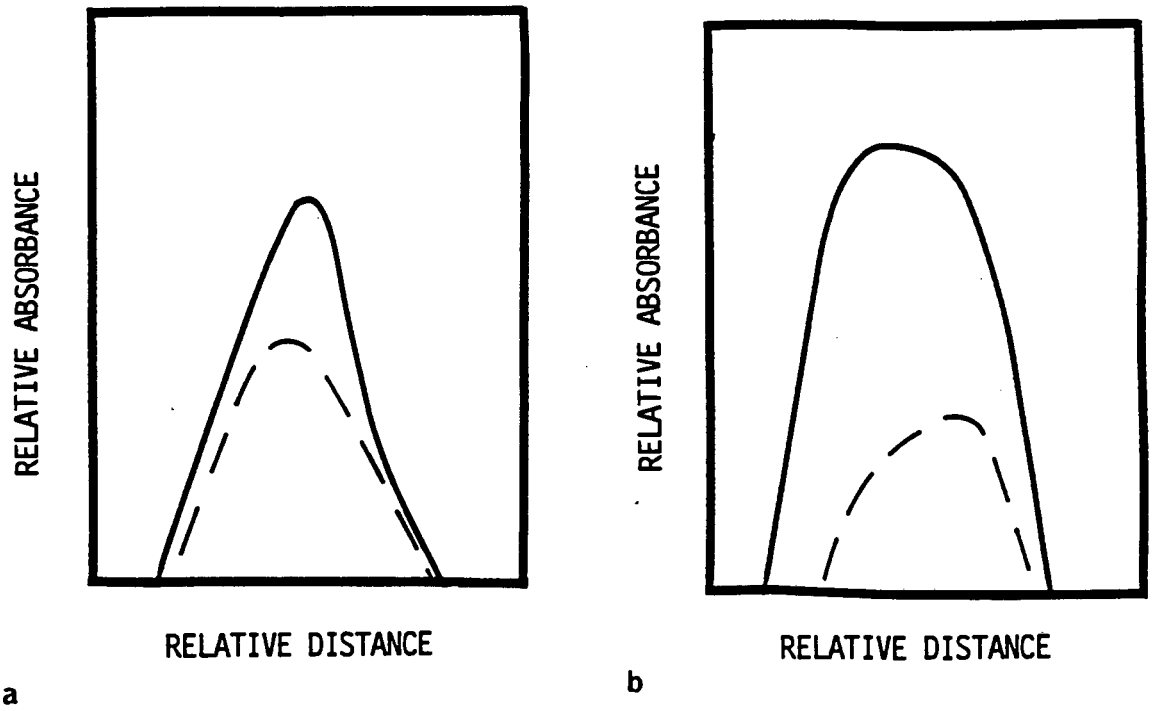


Figure 16.

Comparison of the intensity of the H2A contact near the dyad axis between a: 146 bp and b: 167 bp core particles. The dyad axis (position 0) is at 73 bp in the 146 bp core particle and at 83 bp in the 167 bp core particle. The autoradiograms were densitometrically scanned over the region of this H2A contact (dotted line). This density was compared with that due to the histone H4 contact near position 1.5 (solid line). Position 1.5 is at 88 bp in the the 146 bp core particle and at 99 bp in the 167 bp core particle. Note that the "relative distance" scale (abscissa) reflects different actual distances for the two contacts since migration is dependent on the logarithm of the molecular size.

sites did not indicate a dramatic change in the primary organisation of the 167 bp core particles. Both spots were within 10 bases of major binding regions for that histone (Table 2).
(e) High intensity spots for histone H3 and H2A at 163 and 161 bases respectively.

The histone H4 contacts at about 150 and 163 bases have not been included in figure 15 and Table 2. The reason was that though these spots were clearly visible in some autoradiograms (figure 13c), they were not in others (figure 13a).

The map of the contact sites is depicted in figure 17. The 167 bp core particle retains the essential features of the 146 bp core particle. The evidence for these features is reviewed in Chapter 1. The central region is primarily associated with the tetrameric histones H3 and H4 (though histone H2B does have a weak contact site at about position ± 1) and the outer regions with the dimeric histones H2A and H2B as well as with histone H3. The central 100 bp are bound extensively on both strands in accordance with the higher thermostability of this region (Lindsey and Thompson (1989), Weischet et al. (1978), Simpson (1979)). Regions with less or no observed contact between the DNA and histones are regions of greater DNase 1 accessibility (Lindsey and Thompson (1989), Lutter (1978)). These are at position -2.5, position +5.5 and the 30-40 bases near the 5'-end. The appearance of new H2A and H3 contacts within the 10 bp extension that differentiates the 167 bp from the 146 bp core particle are the most obvious differences between the two particles. The involvement of these histones in binding to the terminal 10 bp of the 167 bp core particle is, however, supported by histone H1 crosslinking studies. Histone H1 has most commonly been found crosslinked to histones H2A and H3 (Bonner and Steadman (1979), Boulikas et al. (1980), Thomas and Khabaza (1980)). Additionally it has also recently been demonstrated that the extended C terminal tail of wheat histone H2A interacts with DNA of the linker region where 8 bp DNA outside of the 167 bp core particle were

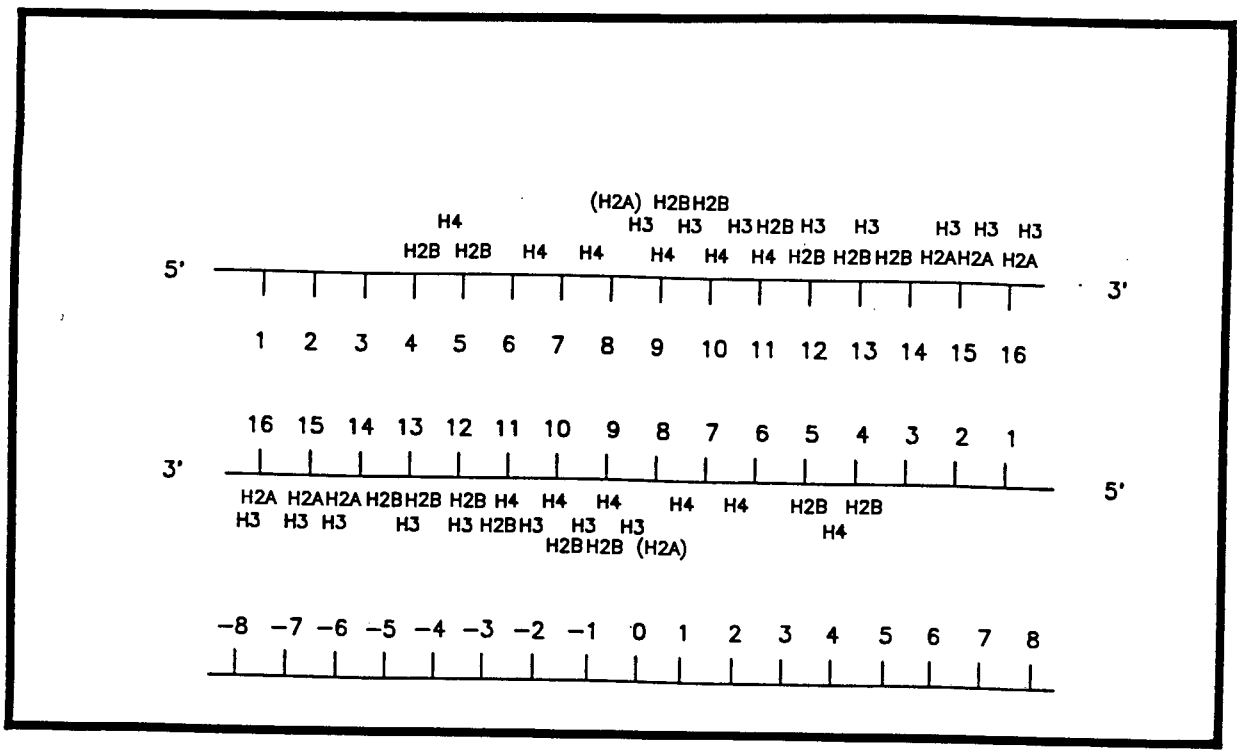


Figure 17.

Map of all the histone contacts on both strands of DNA in the 167 bp core particle.

protected against nuclease digestion (Lindsey et al. (1991)). Another difference between the two core particles was the intensity of the histone H2A contact near the dyad axis. This was markedly reduced in the 167 bp core particle. Mirzabekov and co-workers (Bavykin et al. (1985, 1990), Belyavsky et al. (1980)) have only found this contact in core particles that have been crosslinked after isolation and they have been unable to demonstrate binding at this point both in core particles that have been prepared from crosslinked nuclei or chromatin and in chromatosomes.

These results suggest that the proximity of histone H2A to DNA near the dyad axis of the core particle changes when the DNA attached to the histone octamer is less than 2 full turns. It has previously been demonstrated that tighter DNA binding occurs on trimming the DNA attached to the 167 bp core particle to 146 bp and it has been suggested that this is due to a conformational change in the core particle (Lindsey and Thompson (1989)). It can now be postulated that the position of histone H2A in the core particle depends on both the length of DNA attached and whether histone H1 is present. Thus in unstripped chromatin and the 167 bp core particle this histone is in close proximity with the DNA at the 3' end of the 167 bp core particle, although no binding has been observed in 175 bp chromatosomes (Belyavsky et al. (1980)) possibly due to displacement by histone H1. Removal of histone H1 allows histone H2A to bind to the ultimate 10 bp DNA of the 167 bp core particle. When this 10 bp DNA is removed by digestion to form the 146 bp core particle, histone H2A can obviously no longer bind to it and instead is more closely associated with the next turn of DNA directly beneath site 8 causing the contact observed near the dyad axis. The increase in intensity of the histone H4 contact closest to the dyad axis in the 167 bp core particle is possibly a further indication of a rearrangement of histone-DNA contacts in this region. The location of histone H2A in models based on X-ray crystallographic data (Richmond et al. (1984), Uberbacher and Bunick (1989)) allow this rearrangement to occur and it is of

interest that these models differ in the exact assignment of the position of histone H2A.

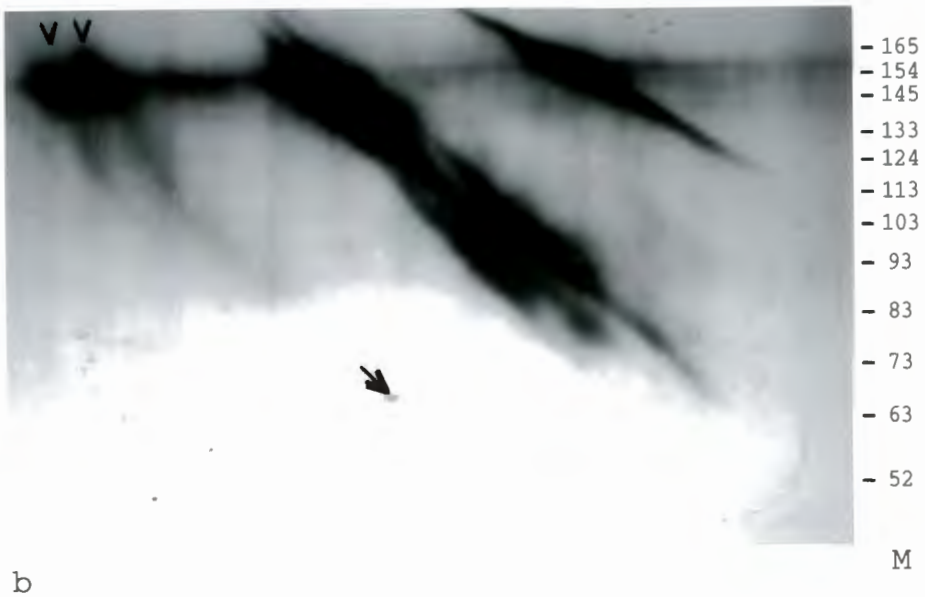
CHAPTER 3

THE PARTIAL IDENTIFICATION OF A PUTATIVE LARGE HISTONE H2B VARIANT

3.1 Introduction

Examination of the two dimensional DNA autoradiograms generated by the zero length DNA:protein crosslinking of 146 and 167 bp core particles always revealed two clearly defined spots well resolved from the histone diagonals. These spots originated from proteins of slower electrophoretic mobility than the core histones and were crosslinked to the 3' end of both core particles (figure 18). The intensity of these spots was seemingly of the same order as the histones; it must be noted, however, that the total histone intensity is the sum of the all the spots represented in the diagonal rather than the intensity associated with any individual spot. Slower migrating proteins associated with core particles could arise from artifacts of the crosslinking process or from the presence of hitherto unknown proteins. Whilst spots due to the attachment of DNA to proteins of seemingly larger molecular size could be obtained if the crosslinking methodology were to cause protein:protein crosslinking concurrent with the protein:DNA crosslinking, it is difficult to envisage how the formation of an electrophilic group could occur on an amino acid during the beta-elimination reaction. Moreover, not only has protein:protein crosslinking not been demonstrated by Mirzabekov and his colleagues but also such crosslinking of histones to one another would result in duplication of the histone diagonals in the second dimension gel rather than yield the defined spots observed. The presence of hitherto unknown proteins associated with chicken erythrocyte core particles is of considerable interest in the light of the recent demonstration of a macroH2A associated with rat liver core particles (Pehrson and Fried (1992)). This protein was almost 3 times the size of H2A and 64 % of the first 120 residues from the N-terminus were identical to H2A.

a



b

Figure 18.

a. Autoradiogram of two-dimensional 7 M urea SDS PAGE of ^{32}P end-labelled single-stranded DNA from crosslinked 146 bp core particles. Second dimension electrophoresis was carried out in a 15 % polyacrylamide gel. The two arrowheads denote the two spots caused by crosslinking of two unknown proteins.

b. Autoradiogram of a two-dimensional 7 M urea SDS PAGE of ^{32}P end-labelled single-stranded DNA from crosslinked 167 bp core particles. As above, except that electrophoresis in the second dimension was carried out using a 12 % gel. The arrow indicates the position of the end-labelled synthetic 68 base polydeoxyribonucleotide.

3.2 Results and discussion

3.2.1 Sizing of the proteins associated with the spots.

An experimental protocol devised by Mirzabekov and Ebralidse (Ebralidse and Mirzabekov (1986), Ebralidse et al. (1988)) made it possible to determine the electrophoretic mobility of the unknown proteins on SDS PAGE. Crosslinked 146 bp core particles stripped of non-crosslinked proteins (section 8.2.1.2) were resuspended and incubated at 70°C in 70 % formic acid 2 % diphenylamine. This procedure resulted in cleavage of the DNA at purine residues. The hydrolysed crosslinked core particles were then treated with calf alkaline phosphatase to remove any 5' phosphates; the remaining DNA was exhaustively trimmed with MNase. This treatment leaves a pyrimidine tag on the amino acid involved in the crosslink (Ebralidse et al. (1988)) which was ^{32}P labelled using [γ ^{32}P] ATP and T4 polynucleotide kinase (section 8.2.5). A five-fold excess (with respect to the protein concentration) of non-crosslinked core particles were subjected to the same protocol as a control. The products of labelling were TCA precipitated and separated on SDS PAGE; a total acid extract of chicken erythrocyte nuclei was used as a standard. The gel was stained with Coomassie Brilliant blue for alignment purposes and autoradiographed (figure 19).

Incorporation of the label into non-crosslinked core particle histones occurred at a very low level suggesting the presence of protein kinase activity in the T4 polynucleotide kinase preparation. Significantly higher incorporation of the label into core histones from crosslinked core particles was observed despite the five-fold lower protein concentration. This indicated that specific phosphorylation had occurred on the pyrimidine tag. The ^{32}P labelled core histones were found to align perfectly with their counterparts in the histone standard demonstrating that the presence of the pyrimidine tag had no effect on histone mobility. Although the unknown

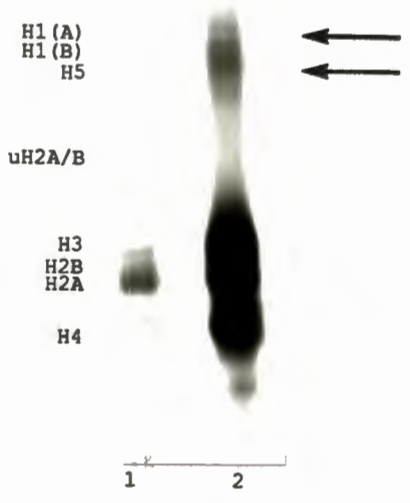


Figure 19.

Autoradiogram of SDS PAGE of ^{32}P labelled proteins from crosslinked and non-crosslinked core particles. Crosslinked (stripped of non-crosslinked proteins (section 8.2.1.1)) and non-crosslinked core particles were treated as described (section 8.2.5). Lane 1: 25 μg histones from non-crosslinked 146 bp core particles; lane 2: 5 μg histones from 146 bp crosslinked core particles (stripped of non-crosslinked histones). The arrows demarcate the region of migration of the unknown proteins.

proteins were not resolved into discrete bands, there was a defined area (demarcated by two arrows) of increased radioactivity on the autoradiogram. This area aligned with histone H5 and the two histone H1 variants. The apparent decrease in intensity of the unknown proteins relative to the core histones in this experiment compared with that observed in the two dimensional DNA gels could be accounted for by the histone bands in figure 19 being a summation of all the spots in their respective diagonals in the two dimensional DNA gels.

The presence of proteins in the crosslinked core particles with a mobility similar to the linker histones on SDS PAGE was intriguing as the linker histones had been stripped from the core particles by gel chromatography in 600 mM NaCl (section 8.1.2). This treatment has been shown (Lindsey and Thompson (1989)) to remove all traces of linker histones from chicken erythrocyte chromatin. Non-crosslinked core particles were therefore re-analysed on SDS PAGE at far greater loadings than previously used in order to determine if any proteins were present with similar electrophoretic mobilities to those of the unknown proteins (figure 20). A doublet could be discerned migrating between histones H1(B) and H5; it is postulated that these two proteins gave rise to the two slower migrating spots observed on the DNA gel. The concentration of the proteins in the doublet were estimated from the staining intensity to be approximately one quarter that of the ubiquitinated histones and therefore of the order of 0.25 % of any core histone (chicken erythrocyte uH2A is of the order of 1.2 % of H2A Goldknopf et al. (1980)). The relatively high labelling intensity in the crosslinking experiments suggests either a close association between these proteins and the 3' end of the DNA or the presence of a highly nucleophilic moiety since the ubiquitinated histones were hardly labelled by comparison.

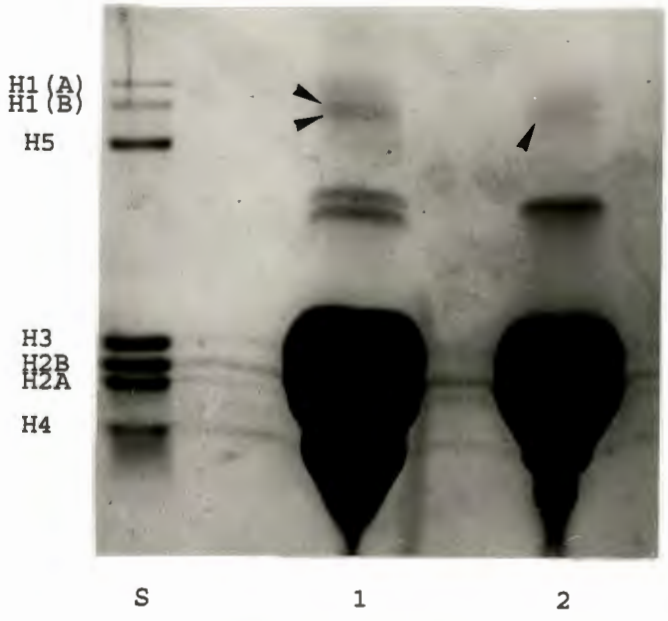
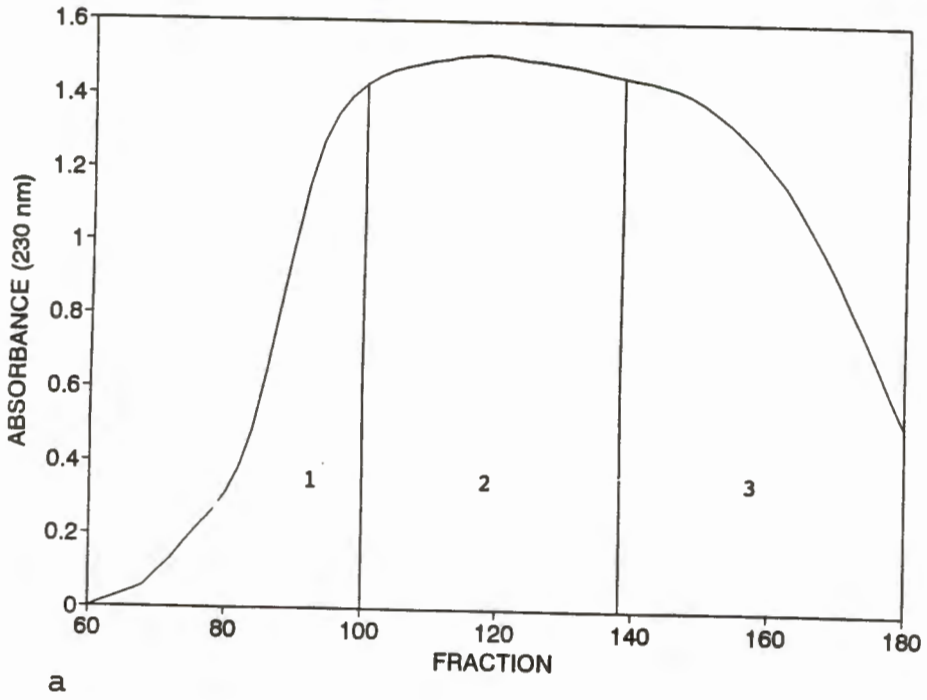


Figure 20.
SDS PAGE of fractions containing the unknown proteins.
Lane 1: 146 bp core particles from chicken erythrocyte which have been chromatographed on Sepharose 4B in 600 mM NaCl 10 mM Tris-HCl pH 7.4 to remove the linker histones (section 8.1.2).
Lane 2: The H2B/H3 fraction from Biogel P60 chromatography of acid extracted core particles (section 8.4.3). The arrows indicate the positions of the unknown proteins. The standard (S) is a total acid extract of chicken erythrocyte nuclei.

3.2.2 Isolation of the slower migrating protein in the doublet

In an initial attempt to purify one or more of these unknown proteins, the protein component of purified chicken erythrocyte core particles (80 mgs DNA) was acid extracted and chromatographed on Biogel P60 in 50 mM NaCl 20 mM HCl (section 8.4.3). The core histones were resolved into an H2A fraction, an H2B/H3 fraction and an H4 fraction. When these fractions were pooled and analysed on SDS PAGE at an extremely high loading (figure 20), a protein with an electrophoretic mobility between histones H5 and H1(B) was found to have co-eluted with the H2B/H3 fraction. This protein had exactly the same electrophoretic mobility as the slower migrating band of the doublet seen on SDS PAGE of non-crosslinked core particles. The very low concentration of this protein in purified core particles precluded core particles as the source and therefore total acid extracted chicken erythrocyte histones were examined for the presence of this protein. A total acid extract of chicken erythrocyte nuclei was therefore separated on Biogel P60 and the H2B/3 fraction analysed on SDS PAGE. A protein with the identical mobility was present in this fraction.

The H2B/H3 Biogel P60 fraction from 900 mg total acid extracted histones was chromatographed on a CM 52 cellulose ion exchange column (section 8.4.2). The eluant was split into 3 approximately equal fractions and analysed on SDS PAGE (figure 21); the unknown protein was present in all 3 fractions. Since uH2B was present in only the first two fractions (albeit in trace quantities in the second), the third fraction was chromatographed on a Biogel P60 column using 7 M urea 2 mM Tris-HCl pH 2 as the buffer (section 8.4.2). This buffer results in complete denaturation of the ubiquitinated histones allowing their separation from their non-ubiquitinated counterparts (see below section 6.2.1); it was hoped that a similar situation might pertain in this case.



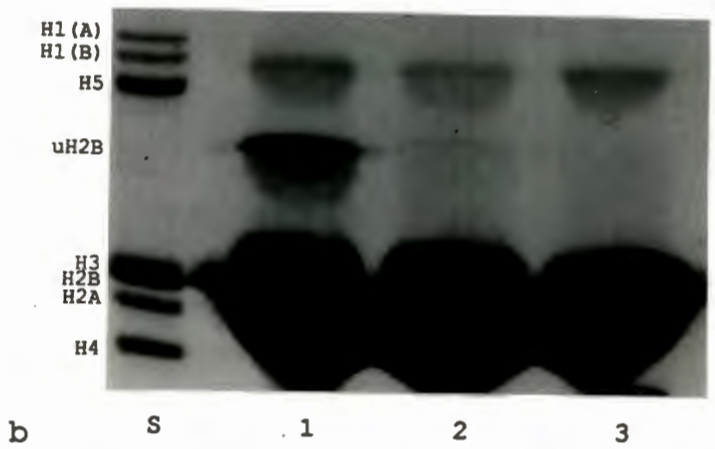


Figure 21.

a. CM 52 cellulose ion exchange chromatography in 6 M urea 50 mM sodium acetate pH 5.4 of the H2B/H3 Biogel P60 fraction of total acid extracted chicken erythrocyte histones. The column was eluted with a linear gradient of 125 - 175 mM NaCl in this buffer. The eluant was split into three fractions (1, 2 and 3).

b. SDS PAGE of the three fractions. Lanes 1, 2 and 3: fractions 1, 2 and 3 respectively. The standard (S) is a total acid extract of chicken erythrocyte nuclei.

The elution profile of this column is presented in (figure 22); SDS PAGE showed that the minor peak eluting prior to the bulk of the material contained only the unknown protein.

The protein was dialysed against water and lyophilised; the yield from 900 mg total acid extracted histones was 20 µg. The absorption spectrum (figure 23) was determined and found to be similar to that of other histones; the A230:A280 ratio was 4:1 suggesting low or no tryptophan content. Moreover, no undue absorption occurred at 260 nm as would be the case if the larger molecular size of this protein was due to nucleic acid addition, for example ADP-ribosylation.

The entire lyophilate (apart from an aliquot taken for SDS PAGE) was subjected to gas phase sequencing (Hewick et al. (1981), Brandt et al. (1984), Lottspeich (1985)). The first 15 amino acids from the amino terminal were unambiguously determined to be identical to the N-terminal sequence of chicken histone H2B (van Helden (1982)). The sequence found was:

```
1     5          10         15
P E P A K S A P A P K K G S K
```

No sequence data could be determined after K15 as the signal from the cleaved amino acid was not distinguishable from background noise. Further characterization of this protein would therefore require an alternative purification approach to obviate the problem of the very low yield experienced.

The unknown protein has tentatively been characterised as a variant of histone H2B and it is proposed to name this protein bH2B (b for big!). The electrophoretic mobility was approximately twice that of histone H2B (histone H3-dimer migrates between H1(A) and H1(B)). This decreased electrophoretic mobility must be due to extensive

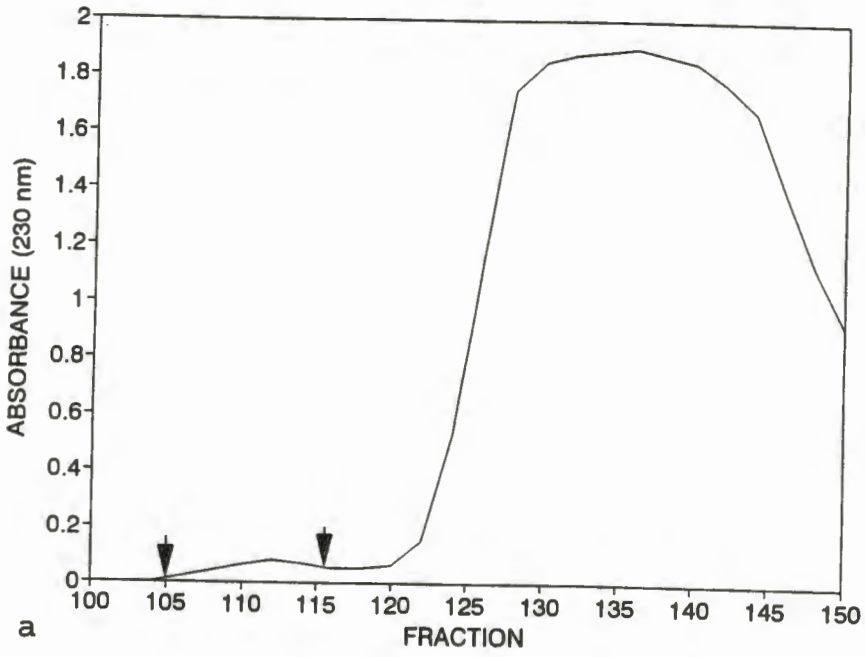


Figure 22.

a. Gel exclusion chromatography of fraction 3 (Fig. 21) on a Biogel P60 column in 7 M urea 2 mM Tris-HCl pH 2.2. The eluant demarcated by the arrows was pooled, dialysed against water and lyophilised.

b. SDS PAGE of the lyophilate. The standard (S) is a total acid extract of chicken erythrocyte nuclei.

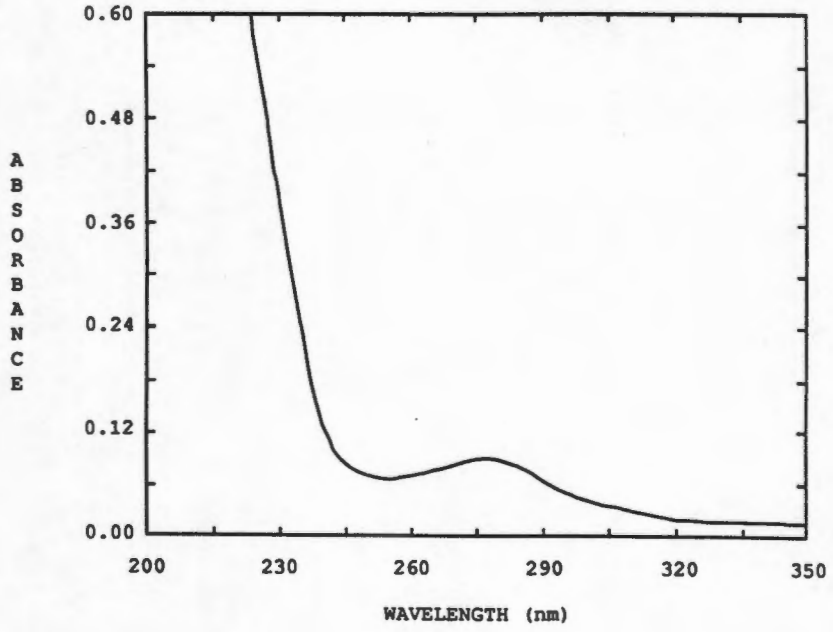


Figure 23.

The absorption spectrum of the unknown purified protein in water.

modification. Possible modifications could be:

1) Poly ADP-ribosylation: histone H2B has been demonstrated to be the major acceptor of mono and poly ADP-ribosylation in dimethyl sulfate treated hepatoma cells (Adamietz and Rudolph (1984)). No band with similar electrophoretic mobility to bH2B was, however, reported. In addition, the spectrum of bH2B revealed no absorbance at 260 nm as would be expected if this modification had occurred.

2) Either approximately double the number of amino acids or a substantial increase in the number of basic amino acids would result in a decreased electrophoretic mobility on SDS PAGE, the latter due to an increased positive charge decreasing the nett negative charge after SDS binding. Possible modifications that could give rise to the results observed could be:
a: a larger histone H2B due to mutation of the stop codon in the gene encoding this protein.
b: histone H2B crosslinked to another protein of an approximately equal size but with a blocked N terminus.
c: dimerisation of histone H2B (this would appear unlikely since natural modifications of this nature have never been reported despite considerable work on histone sequencing at both the protein and DNA level).

The protein appears to be in close proximity to the entry/exit point of the DNA of the core particle. This association has not been directly demonstrated as the identification has relied on comparisons of electrophoretic mobility in SDS PAGE with its concomitant shortcomings. This reliance on electrophoretic mobility was unavoidable as the initial indication of the existence of bH2B was derived from two dimensional DNA gels. A previous experiment to determine the contact site between histone H4 and DNA (Ebralidse et al. (1988)) used a similar approach, however, in that the electrophoretic mobility of the labelled crosslinked trypsinised histone H4 peptide isolated from crosslinked core particles and nuclei was compared with that of purified

histone H4 that was crosslinked to a synthetic dinucleotide and then trypsinised.

CHAPTER 4

UBIQUITINATED HISTONES

4.1 Introduction

Several post translational modifications of histones have been described. These include methylation, acetylation, phosphorylation, ADP ribosylation, glycosylation and ubiquitination (for a list of reviews see: van Holde (1989)). A brief overview of the protein ubiquitin is given in order to facilitate understanding of the possible roles of ubiquinated histones.

4.2 Ubiquitin

Ubiquitin is found both as the free protein or conjugated to a wide variety of proteins in all eukaryotic cells that have been hereto investigated. It appears to be involved in several cellular functions; these have been extensively reviewed (for recent reviews see: Rechsteiner (1988), Finley (1991), Ciechanover and Schwartz (1989), Mayer et al. (1991), Jentsch et al. (1990)). The most clearly understood function of ubiquitin is the labelling of proteins for an ATP-dependent degradation pathway in the cytoplasm. This involves the covalent attachment of the carboxyl terminus of ubiquitin to the ϵ-amino groups of lysine residues of the acceptor protein prior to proteolysis by an ATP-dependent multicomponent protease. This covalent method of attachment is a common theme in all putative functions of ubiquitin.

4.2.1 The structure of ubiquitin

Ubiquitin (molecular weight 8565) was initially isolated by Goldstein et al. (1975) and sequenced by Schlesinger et al. (1975). It is a highly conserved protein with the identical 76 amino acid sequence being found in all animals (Vierstra et al. (1986)). Three amino acid substitutions are present in

yeast (Ozkaynak et al. (1984)); oat ubiquitin differs from yeast and animal by two and three amino acid substitutions respectively (Vierstra et al. (1986)).

The tertiary structure of ubiquitin has been determined by X-ray crystallography at 1.8 Å resolution (Vijay-Kumar et al. (1987)) and by two dimensional proton NMR (Weber et al. (1987)). The protein has a highly compact globular conformation with a marked hydrophobic core. Approximately 90 % of the polypeptide chain is involved in hydrogen-bonded secondary structures. These include a five-strand β sheet, three and one-half turns of α-helix, a short piece of 3_{10}-helix and seven reverse turns. The four residues at the carboxyl terminus (LRGG) are unstructured and protrude from the globular domain of the protein enabling ubiquitin to participate in the formation of ubiquitin-protein conjugates.

4.3 Ubiquitinated histones

4.3.1 Structure

Ubiquitinated H2A (uH2A) was initially known as protein A24. This was the label given by Orrick et al. (1973) to one of the spots on a two-dimensional gel of acid-soluble nucleolar proteins. The nature of the protein was then unknown but subsequently it was shown to be one of the few proteins that decreased markedly in rat liver nucleoli after induction of RNA synthesis by thioacetamide (Ballal et al. (1974)) or partial hepatectomy (Ballal et al. (1975)). Amino acid analysis of the purified protein revealed a similar composition to that of histone H2A (Goldknopf et al. (1975)). Peptide maps contained almost all of the tryptic and chymotryptic peptides of H2A (Goldknopf and Busch (1975)). The protein had both the acetyl-blocked N-terminal sequence of H2A (Goldknopf and Busch (1977)) and an unblocked methionine N-terminal (Goldknopf et al. (1975)) indicating that the protein had a bifurcated polypeptide chain arrangement. The sequence of the first 37 residues from the N-terminus of the unblocked

polypeptide chain (Olson et al. (1976)) was identical to the first 37 residues of ubiquitin (Hunt and Dayhoff (1977)). Subsequently, ubiquitination of histone H2B was also demonstrated (West and Bonner (1980)). Ubiquitin is attached to the ε-amino group of Lys 119 of H2A (Goldknopf and Busch (1977), Goldknopf and Busch (1978)) and to the ε-amino group of Lys 120 of H2B (Thorne et al. (1987)) through its C-terminus. Both of these residues are highly conserved in histones H2A and H2B respectively (Wu et al. (1986)).

UH2A and uH2B have been reported in many eukaryotic species and about 10 % of H2A and 1.5 % of H2B have been reported to be mono-ubiquitinated (Rechsteiner (1988)). Physarum polycephalum is an exception with 6-7 % of both H2A and H2B being ubiquitinated (Mueller et al. (1985)). Low levels of polyubiquitinated H2A and H2B have also been reported in several species (Nickel et al. (1989), Nickel and Davie (1989)). An investigation of the structure of polyubiquitinated H2A from trout liver suggested that there was a chain of ubiquitin molecules attached to the ε-amino group of Lys 119 (Nickel and Davie (1989)). Lys 119 of H2A is accessible (Bohm et al. (1980)) and Lys 120 of H2B inaccessible (Bohm et al. (1982)) to trypsin in the core particle. The ubiquitination site of H2B could also be inaccessible to the enzymes involved in ubiquitination, thus causing the lower levels of H2B ubiquitination.

The enzymatic system for protein ubiquitination in the cytoplasmic ATP-dependent degradation pathway has been well characterised (see reviews: (Rechsteiner (1988), Finley (1991))). The system consists of three enzymes E1, E2 and E3. Briefly, ubiquitin is covalently bound to the ubiquitin activating enzyme, E1, to form an E1-ubiquitin thiol ester in an ATP-dependent reaction. The activated ubiquitin is transferred to one of five ubiquitin-conjugating enzymes, E2. The ubiquitin is then ligated to the acceptor protein in a reaction that usually requires a third enzyme, ubiquitin-protein ligase, E3. Ubiquitination of histones is dependent on

at least part of this system, since uH2A disappears in the mouse cell line ts85, (which has a temperature sensitive mutant of the ubiquitin-activating enzyme, E1) at the nonpermissive temperature (Finley et al. (1984)). *In vitro* studies suggested that only E1 and E2 are required for histones ubiquitination (Pickart et al. (1985), Jentsch et al. (1987)).

4.3.2 Function

Research concerning the biological function of the ubiquitinated histones has concentrated in the areas of cell cycle and transciptional regulation.

4.3.2.1 Metabolism

A rapid turnover of the ubiquitin moiety of uH2A in the nucleus has been reported. Wu et al. (1981) used a combination of pulse chase studies and tryptic peptide mapping to investigate the fate of uH2A in mouse L1210 cells. Whilst the radioactivity of the peptides from the ubiquitin moiety of uH2A decreased rapidly, those from the H2A portion did not. It would therefore seem, in this case, that the attached ubiquitin is not labelling the protein for proteolysis. The 9 hour half-life of the H2A bound ubiquitin was identical to that of free ubiquitin suggesting a rapid equilibrium of these two ubiquitin pools. In addition, a combination of peptide mapping, C-terminal analysis, density labelling and isopycnic centrifugation data indicated that nascent H2A is preferentially conjugated with pre-existing ubiquitin and nascent ubiquitin with pre-existing H2A in MSB-1 cells (Trempe and Leffak (1982)).

4.3.2.2 Effect on nucleosomal structure

Crosslinking and reconstitution studies have not revealed any significant difference between cores containing ubiquitinated histones and those with "standard histones". UH2A crosslinks

to H2B in solution in an equivalent manner to H2A (Martinson et al. (1979)). Histone octamers were separated into H3-H4 tetramers and H2A-H2B dimers using gel chromatography (Hatch et al. (1983)). UH2A and uH2B eluted in the dimer peak demonstrating their involvement in H2A-H2B dimers. In chromatin, H1 could be crosslinked to both uH2A and H2A in a molar ratio determined by the molar ratio of H2A to uH2A (Bonner and Steadman (1979)).

Kleinschmidt and Martinson (1981) reconstituted core particles with uH2A in place of H2A. The presence of two uH2A molecules in the core particle had no discernible effect on either the DNAse 1 digestion pattern or the ability of the core particles to bind the nonhistone proteins HMG 14 and HMG 17.

4.3.2.3 Behaviour in the cell cycle

The ubiquitinated histones have been quantified at various stages in the cell cycle (Matsui et al. (1979), Wu et al. (1981), Mueller et al. (1985)). The disappearance of uH2A in metaphase chromosomes of a chinese hamster cell line (DON) was reported by Matsui et al. (1979). This result was confirmed by Wu et al. (1981) who reported the absence of uH2A and uH2B in chinese hamster ovary cell metaphase chromosomes. Mueller et al. (1985) investigated the precise timing of the disappearance and re-emergence of the ubiquitinated histones in the cell cycle of Physarum polycephalum. Physarum polycephalum was a very suitable system for this investigation as the growth phase is extremely precise with all nuclei in a macroplasmodium dividing within 2-3 minutes in a 9 hour cell cycle. The ubiquitinated histones are stongly present at early prophase, start to disappear at late prophase and have disappeared completely at metaphase. They start to reappear a few minutes later at anaphase. From the findings above, it would appear that ubiquitinated histones are not present in condensed chromatin.

Isopeptidase is an enzyme that cleaves the bond between ubiquitin and a conjugated protein. The increase in the amount of isopeptidase associated with chromatin in mitosis (Matsui et al. (1982)) is in agreement with the postulate of Matsui et al. (1979) that ubiquitination may be involved in the condensation and decondensation of chromatin during the cell cycle. This would require at least two enzymes, one to release ubiquitin from the histones before the onset of mitosis and one to re-ubiquitinate the histones after mitosis. The triggering of mitosis, however, cannot rely solely upon the de-ubiquitination of the histones. As detailed above, when cells from the temperature-sensitive mutant mouse cell line ts85 are placed at the nonpermissive temperature complete loss of uH2A and uH2B occurs but the cells are arrested in the G_2 stage and the chromatin remains dispersed (Yasuda et al. (1981), Finley et al. (1984)). In addition, inhibition of DNA synthesis in T-47D-5 human breast cancer cells had no effect on the levels of uH2A and uH2B (Davie and Murphy (1990)).

4.3.2.4 Transcription

The possible involvement of the ubiquinated histones in transcriptional regulation has recieved intensive investigation. Indeed, several of the earliest reports on the existence of uH2A coupled the disappearance of the protein with the induction of RNA synthesis in rat liver nuclei (Ballal et al. (1974), Ballal et al. (1975). It was therefore suggested that uH2A inhibited transcription (Ballal et al. (1975)). Most evidence since then, however, has indicated that the ubiquitinated histones may rather promote transcriptional activity. Goldknopf et al. (1980), for example, found six times higher levels of uH2A in transcriptionally active erythroid nuclei from phenylhydrazine treated chickens relative to inactive mature chicken erythrocyte nuclei.

In a direct analysis of the proteins associated with active genes and inactive 1.688 satellite DNA in Drosophila, Levinger and Varshavsky (1982) found one uH2A per two nucleosomes in

the active copia and hsp 70 genes and only one per 25 nucleosomes in the satellite DNA. In a similar study, the heavily transcribed dihydrofolate reductase genes in L5178Y-R mouse cells were found to have a high concentration of uH2A near their 5'-ends (Varshavsky et al. (1982)). These results would appear to be strong evidence for the involvement of uH2A with active genes. An investigation by Huang et al. (1986) of the transcribed immunoglobulin kappa gene, however, has suggested "caution in the interpretation" of the data. The experimental protocol devised by Varshavsky and co-workers involved the separation of the slower migrating uH2A containing mononucleosomes from 'standard' mononucleosomes in a two dimensional gel system (Levinger and Varshavsky (1980)). The DNA could then be probed. The identical experiment was carried out by Huang et al. (1986) using a probe for the transcribed immunoglobulin kappa chain gene from mouse plasmacytoma cells. UH2A and uH2B were associated with the slower migrating mononucleosomes as was the immunoglobulin kappa gene. Removal of the ubiquitin from the nucleosomes with isopeptidase (see above) prior to electrophoresis should have changed the mobility of the mononucleosomes containing uH2A and uH2B to that of 'standard' mononucleosomes. The bulk ubiquitinated mononucleosomes did indeed shift but the mononucleosomes containing the immunoglobulin kappa gene did not. This gene is therefore either not associated with the ubiquitinated histones or the isopeptide bond is inaccessible to isopeptidase. It would obviously be of interest to investigate other genes in a similar manner. A further complication in the interpretation of data derived from this protocol was reported by Levinger (1985); he showed that mononucleosomes containing inactive 1.705 satellite DNA showed some enrichment in uH2A.

Quantitation of ubiquitinated histones in chromatin fractions enriched in transcriptionally active sequences has yielded conflicting results. Nickel et al. (1989) found enrichment of uH2A and preferential enrichment of uH2B in nuclease sensitive, low ionic strength soluble chromatin fractions from

chicken erythrocyte and bovine thymus nuclei. Gel exclusion chromatography of the enriched fraction from chicken erythrocyte nuclei produced a fraction 50 times enriched in active β-globin gene sequences (Ridsdale and Davie (1987)). This fraction was enriched 1.5 times in uH2A and four times in uH2B. Some enrichment in uH2A and a substantial enrichment in uH2B in the transcriptionally active tetrahymena macronuclei relative to that in the transcriptionally inert micronuclei has been reported (Nickel et al. (1989)). There was an enrichment of uH2A in the nuclease sensitive, low ionic strength soluble chromatin fraction of myotube cell cultures but there was no corresponding enrichment of active gene sequences (Parlow et al. (1990)). Moreover, when nuclease sensitive murine erythroleukemia chromatin was fractionated by an affinity chromatography methodology into a bound fraction enriched in active globin gene sequences and an unbound fraction, uH2A was equally distributed between the two (Dawson et al. (1991)).

The level of uH2A is unaffected by inhibition of rRNA or heterogenous nuclear RNA (hnRNA) transcription (Ericsson et al. (1986), Davie and Murphy (1990)). UH2B is similarly unaffected by rRNA inhibition but there is an almost complete disappearance of uH2B upon inhibition of hnRNA transcription (Davie and Murphy (1990)). If transcription was allowed to continue, the levels of uH2B returned to normal. Therefore, ubiquitination of H2B is directly dependent upon ongoing transcription. Davie and Murphy (1990) postulated that, as the ubiquitination site of H2B is probably buried in the nucleosome (indicated by it's protection from trypsin in the nucleosome), transcription may open the nucleosome structure allowing ubiquitination to occur. The nucleosome may then be kept in an open conformation during transcription.

After considerable investigation, it would seem that the ubiquitinated histones are not present in condensed chromatin, are not labelled for proteolysis and the ubiquitination of H2B

is dependent upon transcription. The exact function of the ubiquitinated histones is, however, still unclear.

CHAPTER 5

AN ATTEMPT TO INVESTIGATE THE INVOLVEMENT OF THE UBIQUITINATED HISTONES WITH ACTIVE CHROMATIN

5.1 Introduction

It would be informative to isolate DNA that is associated with the ubiquitinated histones and using suitable DNA probes determine whether the ubiquitinated histones are associated with active genes or not. Attempts have been made to isolate nucleosomes that only contain the ubiquitinated histones in place of their non-ubiquitinated counterparts and these have indicated that the ubiquitinated histones were preferentially associated with active chromatin (Varshavsky et al. (1982)), Levinger and Varshavsky (1982)). The fidelity of the nucleosomes has, however, been questioned (Huang et al. (1986)) since mononucleosomes containing the immunoglobulin kappa chain gene still migrated more slowly than bulk nucleosomes after isopeptidase treatment (section 4.3.2.4). The selective isolation of a protein with its associated DNA can be achieved more reliably by non-selectively covalently attaching the protein to its associated DNA and then immunoprecipitating the protein with a specific antibody. The immunoprecipitated protein-DNA complex is then deproteinized and the free DNA probed. The involvement with active chromatin of trout high mobility group protein (HMG) (Blanco et al. (1985)); histone H1 and H5 (Kamakaka and Thomas (1990), Postinikov et al. (1991)) and HMG proteins 14, 17, 1, 2 and E (Postinikov et al. (1991)) has been investigated using essentially this protocol. The crosslink can be introduced by photo-induced coupling using ultraviolet light (Blanco et al. (1985), Kamakaka and Thomas (1990), Postinikov et al. (1991)) or by using the dimethyl sulfate dependent crosslinking protocol devised by Mirzabekov and co-workers (Postinikov et al. (1991)). A comparison between the data of Kamakaka and Thomas (1990) and Postinkov et al. (1991) strongly suggests

that the crosslinking and immunoprecipitation approach is reliable and indepependent of the type of crosslink. Both studies found a very similar depletion (1.5-2 times) of histones H1 and H5 on the active β-globin gene in chicken erythrocyte chromatin relative to non-transcribed chromatin. Kamakaka and Thomas (1990) used an ultraviolet light induced crosslinking protocol and Postinkov et al. (1991) both ultraviolet light induced and the dimethyl sulfate crosslinking protocols. Postinkov et al. (1991) initially fixed their nuclei with formaldehyde prior to dimethyl sulfate crosslinking to avoid displacement of the linker histones and the HMG proteins.

These results combined with the previous successful application of the dimethyl sulfate crosslinking methodology offered the possibility that a similar immunoprecipitation approach could be applied to the question of whether the ubiqitinated histones are associated with active chromatin. The system in which this question was to be investigated was the *Parechinus angulosus* sea urchin embryo. In brief the experimental strategy to be carried out was as follows:

Step 1. after isolation of nuclei from a 16 hour culture of sea urchin embryos, the nuclei would be crosslinked using the dimethyl sulfate methodology. It was considered unlikely that the formaldehyde fixation step would be required as there is strong evidence that the ubiquitinated histones are incorporated in the histone octamer (Chapter 6 and section 4.3.2.2).

Step 2. The crosslinked nuclei would be digested with MNase to generate mainly DNA of dinucleosomal length and the crosslinked complexes would be purified of non-crosslinked proteins on CsCl gradients (Mirzabekov et al. (1989)). It is preferable that the DNA length is short to avoid indirect association between the crosslinked protein and the probe.

Step 3. The purified crosslinked material would be immunoprecipitated with anti-ubiquitin antibodies, the crosslinked proteins removed by proteolysis and the DNA probed. Initially, radiolabelled mRNA purified from transcriptionally active nuclei from a 16 hour culture of sea urchin embryos would be used as the probe (Hames and Higgins (1984)). This would determine if the ubiquitinated histones are associated with actively transcribed sequences. The DNA could also be probed with suitable probes to investigate the possibility that the ubiquitinated histones are involved with chromatin that has been or is about to be transcribed. The level of enrichment would be quantitated by the amount of probe binding to the immunoprecipitated DNA relative to that binding to DNA from crosslinked nuclei of a 16 hour culture of sea urchin embryos that had not been immunoprecipitated.

An absolute requirement for this experimental protocol is an antibody of high affinity and specificity. This, unfortunately, was not achieved. Attempts to overcome the limitations of the antibodies raised against conjugated ubiquitin have been detailed and discussed.

5.2 Results and Discussion

5.2.1 Preparation of a polyclonal antibody against conjugated ubiquitin

Ubiquitin is a highly conserved polypeptide (the primary sequence of 76 amino acids is identical in all mammals) and is therefore a poor immunogen. A common technique used to increase the immunogenicity of a molecule is to couple the molecule to a highly immunogenic carrier protein. This approach has been successfully used in rabbits to raise antibodies against ubiquitin (Hershko et al. (1982), Haas et al. (1985)). The reported optimal conditions were 18 ubiquitin molecules coupled per bovine γ globulin molecule using glutaraldehyde as the cross-linking reagent. The antisera obtained, however, were specific for free ubiquitin and had much lower affinity for conjugated ubiquitin. Antibodies that

recognised conjugated ubiquitin as well as unconjugated ubiquitin could only be obtained if the ratio of ubiquitin to Bovine γ globulin was reduced to 4.6:1 and the whole complex heat denatured in 2 % SDS prior to immunization (Hershko et al. (1982), Haas and Bright (1985)).

Accordingly, 6 rabbits were immunized using the exact protocol described by Haas and Bright (1985) (section 8.3.1). The titres of the various serums were monitored with an antibody capture assay using immobilised uH2A (section 8.3.1.3). The highest titre was obtained after the fifth injection in all the rabbits but the individual rabbits had different titres (figure 24). The antisera from the fifth bleed of the rabbit with the highest titre (1:10000) was used for the following work.

5.2.2 Attempts to immunoprecipitate ubiquitinated histones

A preliminary immunoprecipitation was carried out using standard methodology (Harlow and Lane (1988)) (section 8.3.3). The immunoprecipitation was performed using a total acid extract of calf thymus nuclei as this was the closest representation of the task that the anti-uH2A antibodies were to be used for. The immunoprecipitated products were separated by SDS PAGE and autoradiographed as analysis of immunoprecipitation products with SDS PAGE is a precise method for determining the specificity of an antibody (Postinikov et al. (1991)). Two critical problems for further use of the antibody were revealed in this autoradiogram (figure 25).

One problem was that no enrichment of the ubiquitinated histones present in total acid extracted calf thymus histones was detected after immunoprecipitation with anti-uH2A serum. In contrast, an equivalent amount of antiserum against a nerve growth factor extracted from the South African puffadder, *Bitis arietans*, could successfully immunoprecipitate the nerve growth factor. This suggested that the antibodies present in the anti-uH2A serum had low affinities for the ubiquitinated

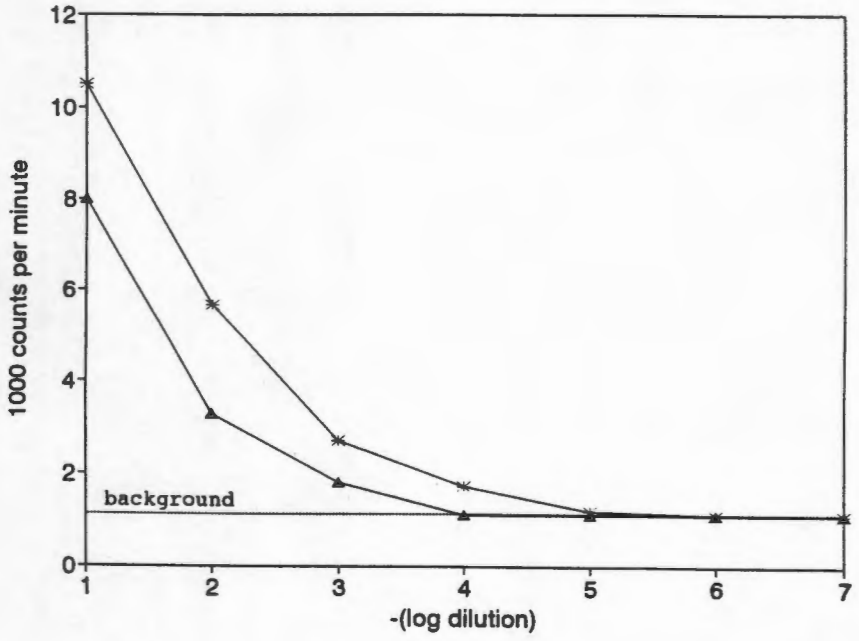


Figure 24.

Titration curves from the antiserum of the fifth bleed of the two rabbits with the highest titres. The immunoassays were carried out as described in section 8.3.1.3. The background value was obtained from the assay of a 10 times dilution of pre-immune serum of the rabbit represented by stars.

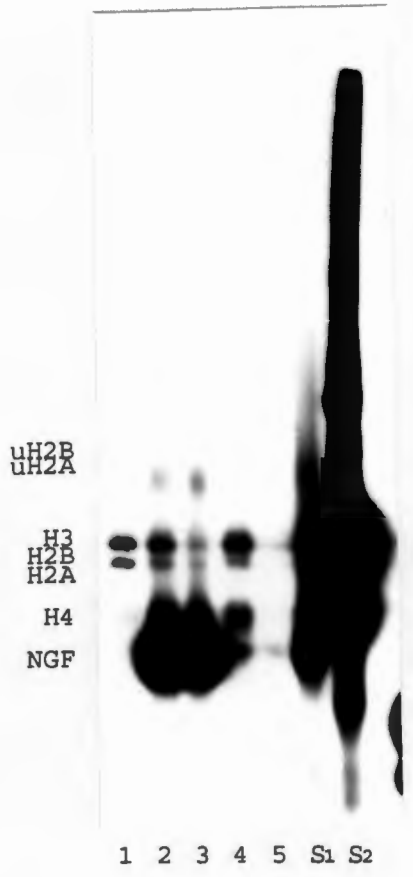


Figure 25.

Autoradiogram of SDS PAGE of immunoprecipitated (section 8.3.3) ^{125}I labelled proteins. Immunoprecipitation was carried out with the immune serum stated against the following antigens:
Lane 1: 1 μl pre-immune serum against 1 μg ^{125}I labelled total acid extracted calf thymus histones.
Lane 2: 1 μl anti-nerve growth factor serum against 1 μg ^{125}I labelled total acid extracted calf thymus histones together with 0.02 μg ^{125}I nerve growth factor (NGF).
Lane 3: 1 μl anti-nerve growth factor serum against 0.02 μg ^{125}I nerve growth factor.
Lane 4: 1 μl anti-uH2A serum against 1 μg ^{125}I labelled total acid extracted calf thymus histones.
Lane 5: Protein A-Sepharose-4B CL (no serum) against 1 μg ^{125}I labelled total acid extracted calf thymus histones.
The standards are 0.02 μg ^{125}I nerve growth factor (S_1) and 1 μg ^{125}I labelled total acid extracted calf thymus histones (S_2).

histones. The second problem was that the core histones bound nonspecifically to the antibodies. This phenomenon was not restricted to the antibodies in the anti-uH2A serum but occurred with both the anti-nerve growth factor serum and the pre-immunization serum. No nonspecific binding to Protein A-Sepharose CL-4B was detected. As the ubiquitinated histones are always a minor component of the histones, of the order of 2 %, nonspecific binding of bulk histones to the antiserum would have to be greatly reduced to allow significant enrichment of the ubiquitinated histones. Several strategies were therefore employed in an attempt to circumvent these two problems.

5.2.2.1 Attempts to reduce nonspecific binding of bulk histones and increase the efficiency of specific binding.

A simple solid phase immunoassay was set up to allow the screening of the various procedures used for the reduction of nonspecific binding (section 8.3.2). This involved spotting the purified individual core histones (depleted of ubiquitinated histones) and uH2A onto nitrocellulose, after which the nitrocellulose was dried at 50°C and probed with anti-uH2A antibodies. The blots were then extensively washed and bound antibody detected with ^{125}I labelled protein A. 75-80 % of ^{125}I labelled core histones and uH2A remained bound to the nitrocellulose after washing in 0.01 M TBS pH 7.4 with or without 0.2 % SDS. The immunoassay allowed detection of 0.5 picomoles of uH2A and this was roughly comparable with the sensitivity reported for anti-conjugated ubiquitin antibodies by Haas and Bright (1985).

When the solid phase immunoassay was carried out under the same conditions as the immunoprecipitation (2 % BSA 0.01 M TBS pH 7.4) (section 5.2.2), nonspecific binding to the core histones was clearly visible (figure 26a). The level of nonspecific binding was approximately only 10 times less than that of specific binding to uH2A. The incubation of the

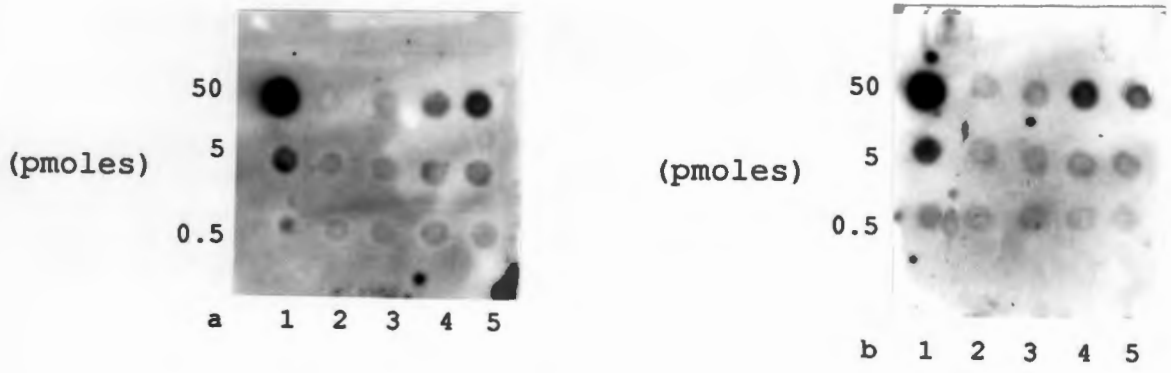


Figure 26.
Solid phase immunoassay (section 8.3.2) to determine the extent of anti-uH2A serum binding to purified uH2A (1), H2A, H2B, H4 and H3.

a. Autoradiogram of immunoassay carried out in 2 % BSA 0.01 M TBS pH 7.4. Binding of ^{125}I labelled Protein A could not be detected under these conditions.

b. Autoradiogram of immunoassay carried out in 2 % BSA 2 M NaCl 0.2 % SDS 1 % Triton X-100 1 % sodium deoxycholate 0.01 M Tris-HCl pH 7.4.

antibody and washing of the antibody-antigen complex in the immunoassay were then performed in different ionic strength solutions incorporating various detergents in an attempt to find conditions that reduced nonspecific core histone binding. (see Table 3)

Table 3 Incubation and washing solutions .

	BSA	NaCl	SDS	Triton-X-100	Na deoxy-cholate	nonfat dry milk
	% w/v	[M]	% w/v	% v/v	% w/v	% w/v
1.	2	0.15	0	0	1	0
2.	2	0.15	0	1	0	0
3.	2	0.15	0.2	0	0	0
4.	2	2	0	0	0	0
5.	2	2	0.2	1	1	0
6.	5	0.15	0	0	0	0
7.	0	0.15	0	0	0	5
8.	0	0.15	0	0	0	2

The buffer used throughout was 10 mM Tris.HCl pH 7.5.

No diminution of specific or nonspecific binding was found to result from incubation and washing in solutions 1-6. The autoradiogram of the immonoassay carried out in solution 5 showed that even a combination of high ionic strength and cationic and non ionic detergents had no discernible effect (figure 26b). Solutions 7 and 8 (commonly known as Blotto) caused a complete loss of both nonspecific and specific binding.

Two alternative approaches to reduce nonspecific binding were also unsuccessful. These were either passing the anti-uH2A serum over a histone H3-Sepharose 4B column (section 8.3.3.3) or affinity purifying the anti-uH2A antibodies on an ubiquitin-Sepharose 4B column (section 8.3.3.2). In the former

case it was hoped that a subset of antibodies were responsible for nonspecific binding and would be adsorbed onto H3 (Postinikov et al. (1991)) (H3 exhibited the highest nonspecific binding (figure 26) and in the latter case that the affinity purified antibodies would have decreased nonspecific binding. H3-Sepharose 4B treated anti-uH2A serum was assayed at the same dilution (1/1000) as that used for the untreated anti-uH2A serum and affinity purified antibody at 10 μg purified antibody/ml 1 % BSA 0.01 M TBS pH 7.4. In both cases the levels of specific and nonspecific binding were very similar to that found previously.

Attempts were made to alter the immunoprecipitation conditions such that increased specific binding might occur and thereby reduce the relative nonspecific binding.
The following was therefore carried out:
(i) The period of antibody incubation during immunoprecipitation was increased to 12 hours and carried out at 4°C as antibody-antigen interactions have been reported to have lower equilibrium constants at lower temperatures (Anderson et Blobel (1983)).
(ii) Either a 10 fold increase in the anti-uH2A serum concentration or 30 μg of affinity purified antibody (equivalent to a 100 fold increase in anti-uH2A serum) were used in an effort to drive the equilibrium over towards more bound antigen. (30 μg of affinity purified antibody was used in the previously reported immunoprecipitation of ubiquitin conjugates from rabbit reticulocytes (Haas and Bright (1985)).

(iii) The total acid extract of calf thymus nuclei was boiled in 1 % SDS prior to immunoprecipitation as the ubiquitin had been heat denatured in 2 % SDS before immunization of the rabbits. Immunoprecipitation was performed in 0.2 % SDS 1 % Triton X-100 0.15 M NaCl 50 mM Tris-HCl pH 7.4 (Anderson et Blobel (1983)). It was postulated that more epitopes might thereby be revealed to the antibodies.

No alteration in the specific binding to nonspecific binding was achieved with any of these modifications or possible combinations thereof.

The antibody described here appeared to behave similarly to antibodies described in the literature. UH2A could be detected down to similar levels (0.5 pmoles) when uH2A was dot blotted on nitrocellulose (Haas and Bright (1985)); the titre of the immune serum (1:10000) was greater than that reported by Meyer et al. (1986) of 1:1200. Although Hershko et al. (1982), achieved immunoprecipitation of ubiquitin conjugates from rabbit reticulocytes, the aim of their investigation was to quantitate the turnover through ubiquitin dependent proteolysis of protein conjugated to ubiquitin and no data was presented on the immunoprecipitation of ubiquitinated histones. There was evidence, though, that immunoprecipitation might require multiply ubiquitinated conjugates as only ubiquitin that had been crosslinked and denatured in SDS could compete for binding in the immunoprecipitation assay. It now appears that ubiquitin-dependent proteolysis requires multiple ubiquitination of the target protein (Finley (1991)).

CHAPTER 6

THE CORE PARTICLE RECONSTITUTED WITH uH2A OR uH2B

6.1 Introduction

The effect of ubiquitination of H2A on the core particle structure has been investigated by reconstitution methodology and nuclease digestions (Kleinschmidt and Martinson (1981)). The presence of uH2A was found to have little influence on the core particle structure. Two features of H2B ubiquitination indicated that uH2B may have a more pronounced effect on core particle structure. These are the inaccessibility of the H2B ubiquitination site (lys 120) in nucleosomes to trypsin (Bohm et al. (1982)) compared with the accessibility of the H2A site (lys 119) (Bohm et al. (1980)) and that uH2B, unlike uH2A, has been coupled to ongoing transcription (Davie and Murphy (1990), Ericsson et al. (1986)). As stated above (section 4.3.2.4), Davie and Murphy have therefore suggested that the H2B ubiquitination site is buried in the nucleosome but becomes accessible during transcription due to unfolding. The resultant ubiquitination has been postulated to hold the nucleosome in an open conformation. It was therefore of interest to investigate the effect of H2B ubiquitination on core particle structure using reconstitution methodology and nuclease digestion. The effect of uH2A was also reinvestigated since a different reconstitution methodology was employed to that used previously (Kleinschmidt and Martinson (1980)).

6.2 Results and Discussion

6.2.1 Purification of ubiquitinated histones

The ubiquitinated histones uH2A and uH2B were isolated from calf thymus nucleoprotein (section 8.4.1). UH2A co-fractionated with the H2A/H3 fraction on a Biogel P60 column in 50 mM NaCl 20 mM HCl and uH2B co-fractionated with the

H2B/H3 fraction (section 8.4.2). The histones are present as random coils under these conditions and behave as much larger proteins. Ubiquitin, however, remains folded and therefore contributes minimally to the effective size of the attached histone (Thorne et al. (1987)). The H2B/H3 and the H2A/H3 fractions were individually pooled and applied to a CM 52 cellulose ion exchange column in order to enrich the ubiquitinated histones in these fractions and to remove high molecular weight contaminants (section 8.4.2). The ubiquitinated histones eluted at lower ionic strength than the core histones presumably due to the presence of the less basic ubiquitin. The ubiquitinated histones, however, were still substantially contaminated by core histones and it was necessary to further purify them using gel exclusion chromatography in the presence of 7 M urea (Hunter and Carey (1985), Thorne et al. (1987)) (section 8.4.2). The ubiquitin conjugate is reversibly denatured by high urea concentrations (Cary et al. (1980)) and the ubiquitinated histones are therefore eluted prior to the core histones (figure 27).

The identity of the purified ubiquitinated histones (figure 27) was confirmed by amino acid analysis (Klapper (1982)) and gas phase protein sequencing (Hewick et al. (1981), Brandt et al. (1984), Lottspeich (1985)). The protein that migrated slower on SDS PAGE was identified as uH2B since it yielded equivalent quantities of two amino acid residues at each cycle of the Edman degradation; these were Met and Pro, Gln and Glu, Ile and Pro. These amino acids correspond with the first three residues of ubiquitin (M-Q-I) and calf thymus H2B (P-E-P). The protein that migrated faster on SDS PAGE was identified as uH2A since it yielded only the sequence Met Gln Ile corresponding to the first three amino acids of ubiquitin. No sequence from the histone component of uH2A was detected as the N-terminus of H2A is acetylated and is thereby blocked to the Edman chemistry. Amino acid analysis of the faster migrating protein (Table 4) showed a composition that coincided with that of ubiquitin together with calf thymus H2A.

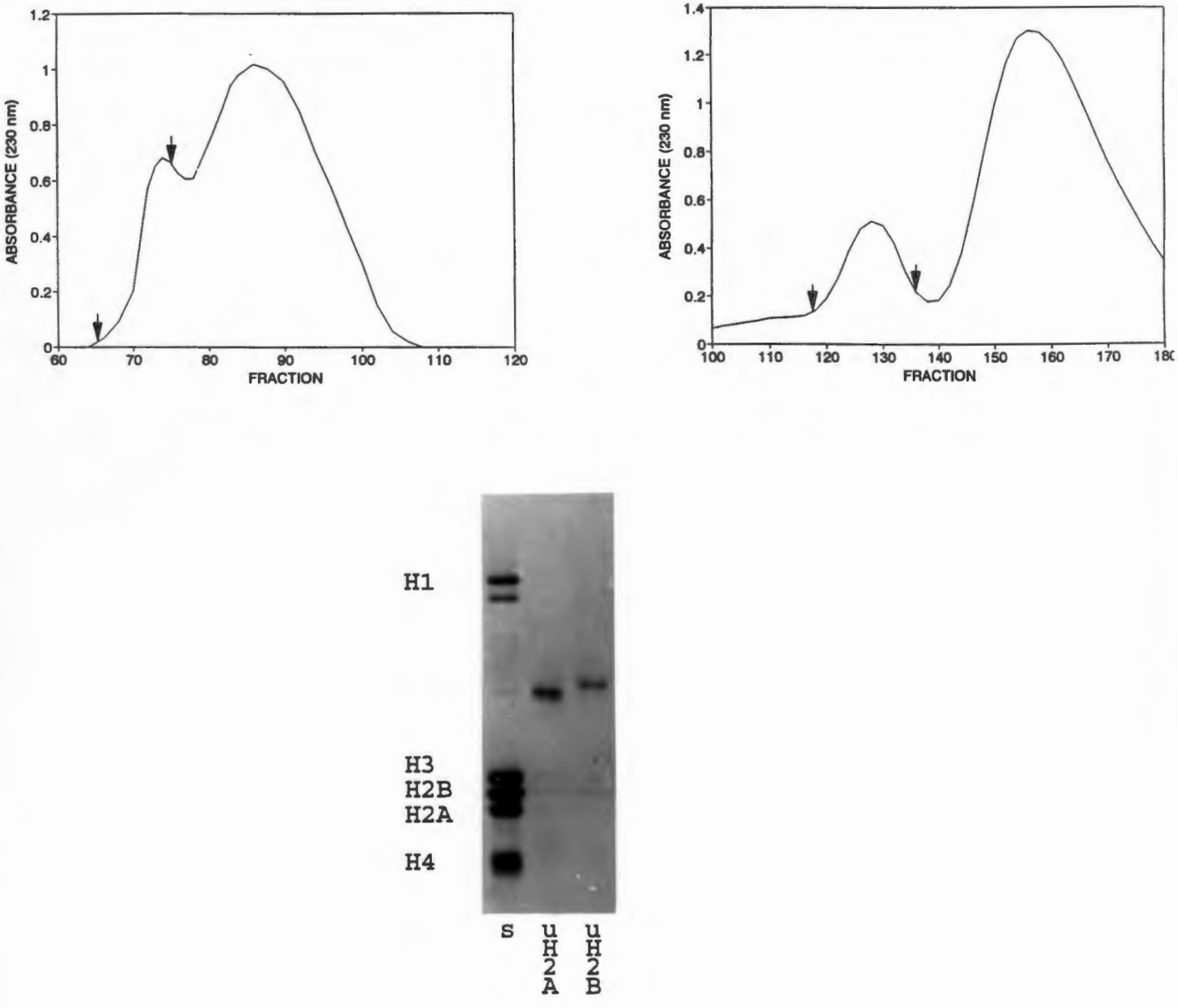


Figure 27.

Biogel P60 gel exclusion chromatography in 7 M urea 2 mM Tris-HCl pH 2.2 of pooled fractions containing enriched uH2B or uH2A from the CM 52 cellulose ion exchange column (see figure 21 for this step carried out for the purification of bH2B). The peaks demarcated by arrows on both elution profiles were pooled, dialysed against water and lyophilised.
top left: uH2A
top right: uH2B
lower. SDS PAGE of lyophilised purified ubiquitinated histones. The standard (S) is a total acid extract of calf thymus nuclei.

Table 4.
Amino acid analysis of uH2A

Amino acid	Moles found	Calculated
Asp	14.6 (15)	16
Thr	10.3 (10)	11
Ser	7.5 (7)	8
Glu	25.1 (25)	23
Pro	10.7 (11)	8
Gly	19.9 (20)	19
Ala	21.3 (21)	20
Cys	0.0	0
Val	12.0 (12)	12
Met	0.2	1
Ile	12.1 (12)	13
Leu	24.6 (25)	25
Tyr	2.7 (3)	4
Phe	3.5 (3)	3
Trp	0.0 (0)	0
His	6.4 (6)	4
Lys	20.9 (21)	21
Arg	17.4 (17)	16
TOTAL	209.1 (209)	204

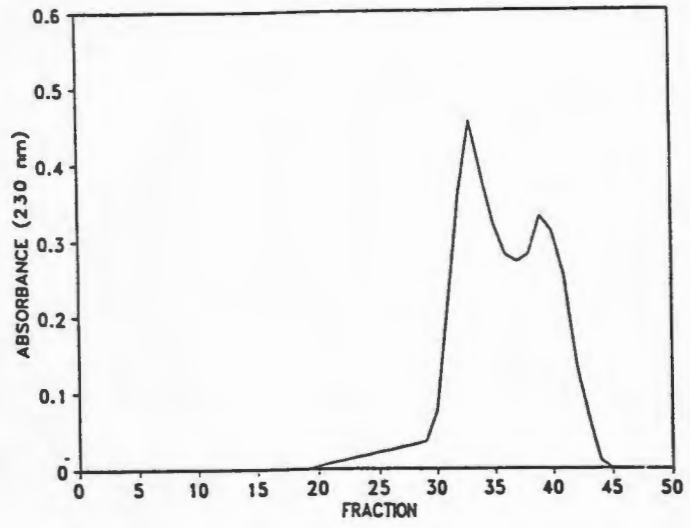
6.2.2 Reconstitution of histone octamers

Three types of octamer were reconstituted (section 8.5.1). These were an octamer with core histones only, an octamer with H2A replaced by uH2A and an octamer with H2B replaced by uH2B. These were reconstituted from stoichiometric amounts of their individual acid-denatured components by dialysis against 2 M

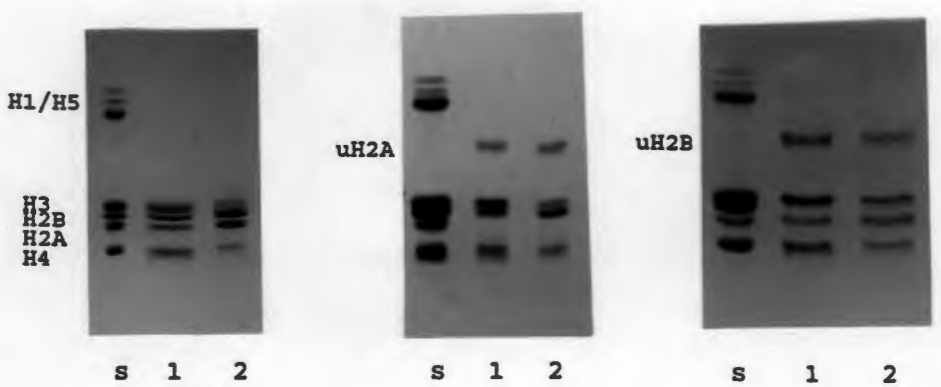
NaCl (von Holt et al. (1989)). This methodology using core histones produces octamers that crystallize in the same form of helical tubes as natural octamers (Greyling et al. (1983)) (natural octamers being defined as those extracted from chromatin with 2 M NaCl). The reconstituted octamers were purified by gel exclusion chromatography on a Sephacryl S300 column (figure 28a) and had identical elution profiles. The histones in the faster migrating peak were analysed by SDS PAGE and found to contain equivalent quantites of histones H3, H2B or uH2B, H2A or uH2A and H4 (figure 28b-d). This peak was thus identified as containing the reconstituted octamer. The efficiency of reconstitution was in the range 25-40 %.

6.2.3 Reconstitution of core particles

The various octamers were assembled onto long DNA (> 1000 bp) using the poly(glutamic) acid reconstitution methodology (Lindsey et al. (1991), Retief et al. (1984), Stein et al. (1979)) (section 8.5.2). The polyanion is thought to transiently stabilize the octamer before octamer binding to the DNA occurs (Retief et al. (1984), Stein et al. (1979)). The core particles obtained by this methodology have been shown to be indistinguishable from natural core particles in their susceptibility to DNase 1 (Retief et al. (1984)). Trial digestions with MNase, to determine the optimal time to obtain homogeneous core particles containing 146 bp of DNA, were identical for the three types of core particles (figure 29). The digestion proceeded through 167 bp and 157 bp intermediates as reported for natural (Lindsey and Thompson (1989)) and reconstituted core particles (Lindsey et al. (1991)). The core particles, purified on isokinetic 5-20 % sucrose gradients, migrated as sharp co-incidental gaussian peaks (figure 30a). The purified core particles had stoichiometric amounts of their constituent histones and discrete DNA lengths of 146 bp (figure 30b-e). Core particle yield was in the range 7-10 %. These results demonstrated that H2A could be replaced by uH2A in the core particle with no obvious structural effect in agreement with results of



a



b c d

Figure 28.

a. Sephacryl S300 chromatography of the products of octamer reconstitution of chicken erythrocyte histones H3, H2A and H4 together with calf thymus uH2B.

b - d. SDS PAGE of the proteins present in the first eluting peak, lanes 1, and the second eluting peak, lanes 2, from Sephacryl S300 chromatography of the products of octamer reconstitution from chicken erythrocyte core histones (b), with uH2A replacing H2A (c) and with uH2B replacing H2B (d). The standard (S) is a total acid extract of chicken erythrocyte nuclei.

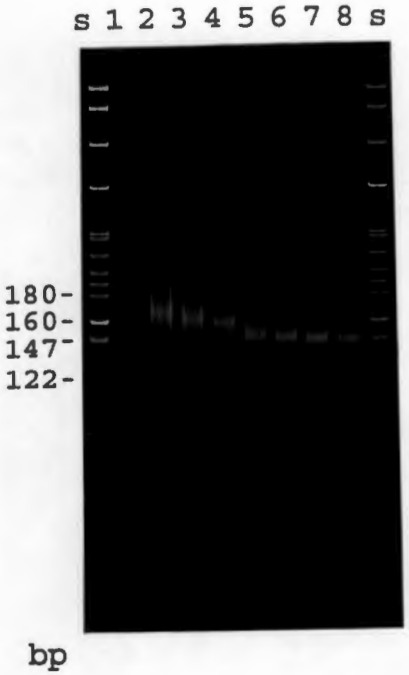
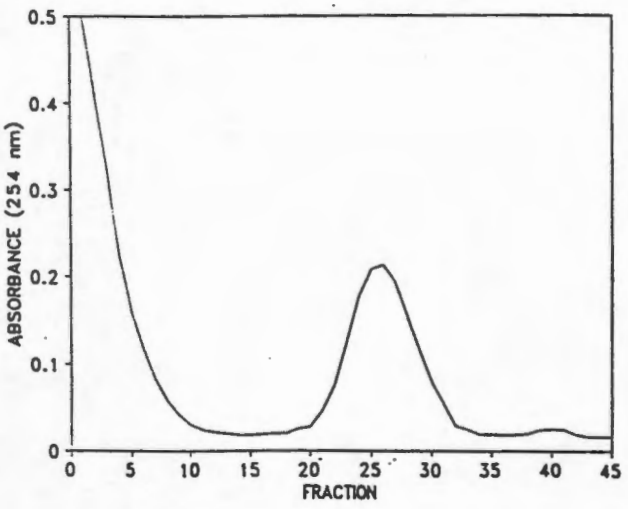
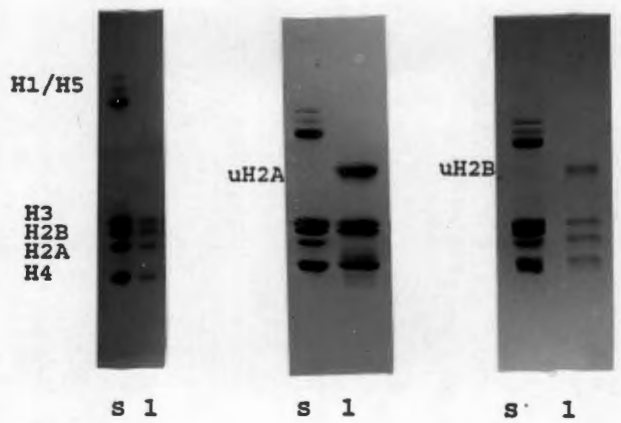


Figure 29.

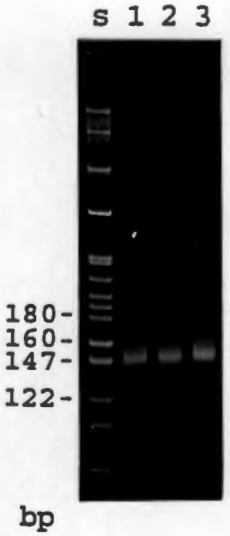
6 % non-denaturing PAGE of the products of MNase digestion of uH2B hybrid octamers (figure 26d, lane 1) assembled onto long DNA. The digestion was stopped at 0, 0.5, 1, 2, 5, 10, 20 and 40 min (lanes 1 - 8). The standard (S) is a *Hpa*II digest of pBR 322.


0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE (254 nm)
0 5 10 15 20 25 30 35 40 45
FRACTION

a


H1/H5
H3
H2B
H2A
H4
uH2A
uH2B
s 1
s 1
s 1

b c d


s 1 2 3
180-
160-
147-
122-
bp

e

Figure 30.

a. Isokinetic sucrose gradient ultracentrifugation profile of uH2B hybrid core particles.

b - d. SDS PAGE of histones present in control (b) and hybrid (uH2A: c, uH2B: d) core particles purified by sucrose gradient centrifugation. The standard (S) is a total acid extract of chicken erythrocyte nuclei.

e. 6 % non-denaturing PAGE of DNA present in control, uH2A and uH2B hybrid core particles (lanes 1 - 3 respectively). The standard (S) is a *Hpa*II digest of pBR 322.

Kleinschmidt and Martinson (1980); similarly H2B could be replaced by uH2B.

6.2.3.1 DNase 1 digestions of reconstituted core particles

The conformations of the core particles were examined in greater detail through partial DNase 1 digestion (section 8.5.2.1). The cutting pattern produced by DNase 1 digestion of core particles results in a 10 bp period due to the DNA being wrapped around the outside of the core histone octamer in a left-handed superhelix (Noll (1974), Lindsey and Thompson (1989)). The sites of cleavage are not uniformly susceptible to DNase 1 attack probably due to sensitivity of the nuclease to the local conformation of the DNA. The positions of maximum protection from cleavage (most comprehensively defined by Lutter (1980)) have been correlated with sharply bent regions of the DNA (Richmond et al. (1984)). It has been postulated that these sharp bends are due to interaction of the DNA with basic regions of the histone octamer (Manning et al. (1989), Ebralidse et al. (1988)). A comparison of the DNase 1 cutting patterns obtained from the ubiquitinated core particles with that from non-ubiquitinated (control) core particles may therefore reveal structural differences induced by the presence of the ubiquitin.

The products of DNase 1 digestion of the 5'-end-labelled core particles were separated by denaturing PAGE (section 8.5.2.1) (figure 31) and were densitometrically scanned (figure 32). The cutting patterns obtained for both the standard and the uH2A core particles showed little difference and are in general agreement with those published previously (Kleinschmidt and Martinson (1981), Lutter (1978)), Lindsey and Thompson (1989)). Surprisingly, since the ubiquitination site in histone H2B is not exposed in the core particle (Bohm et al. (1982)), the DNase 1 cutting pattern for the uH2B core particles was very similar to those of both standard and uH2A core particles. It would therefore appear that complete H2B ubiquitination had no effect on the conformation of the core

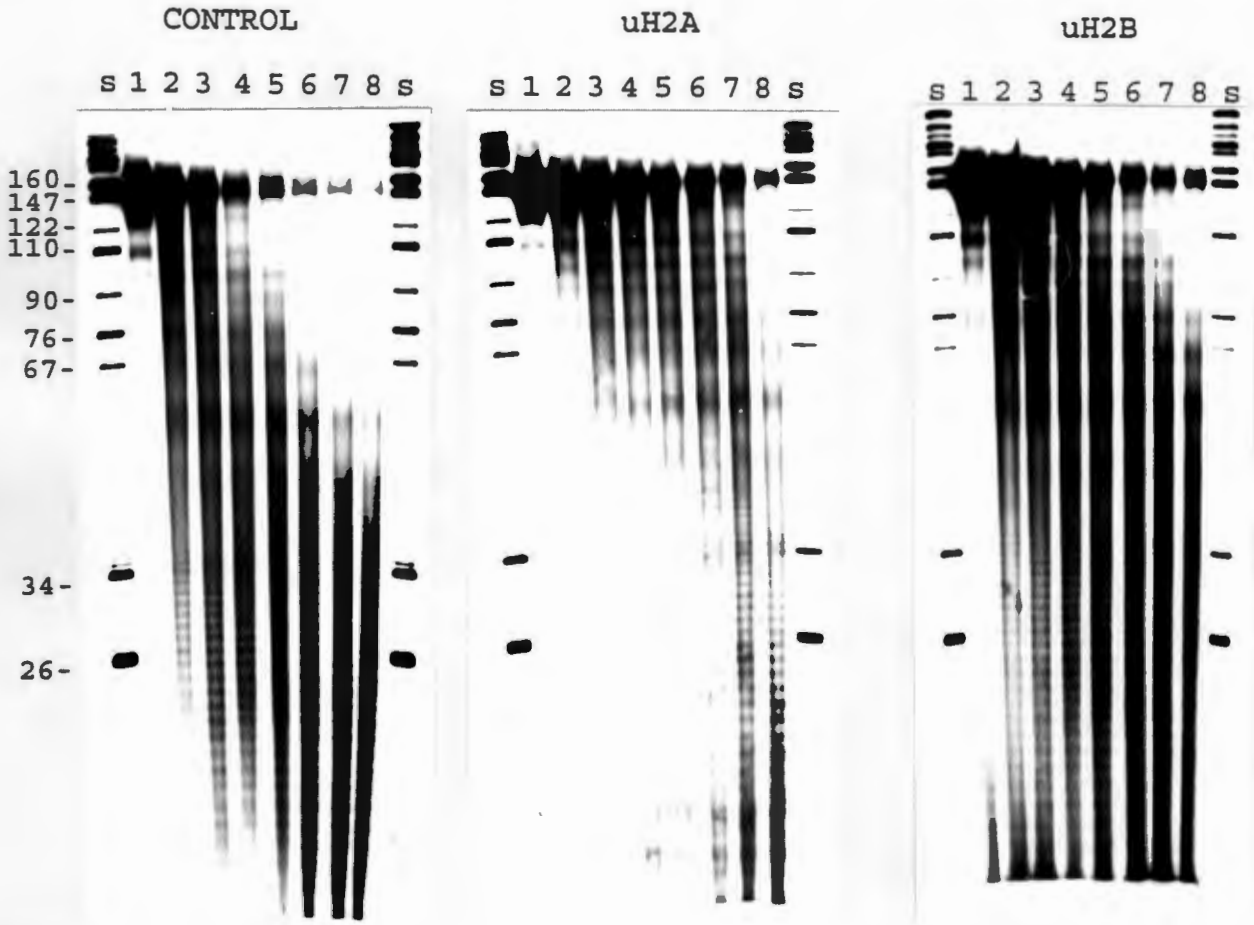
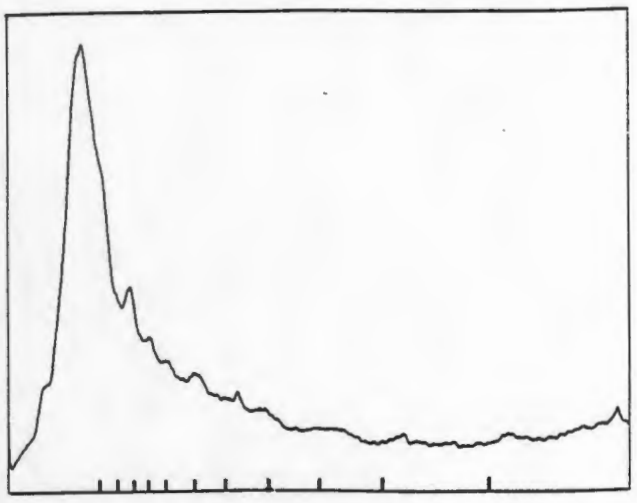
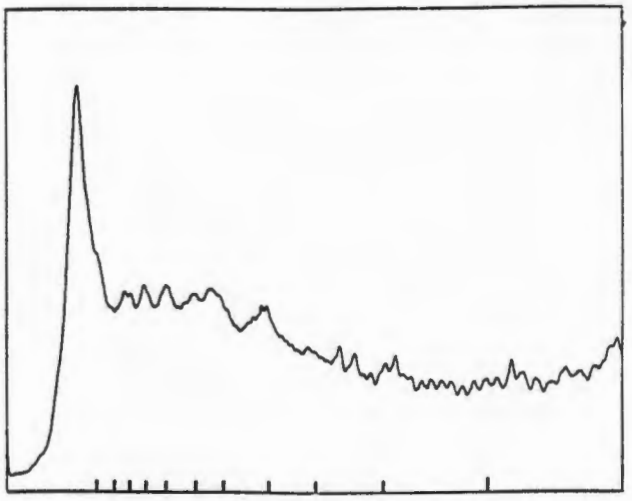


Figure 31.

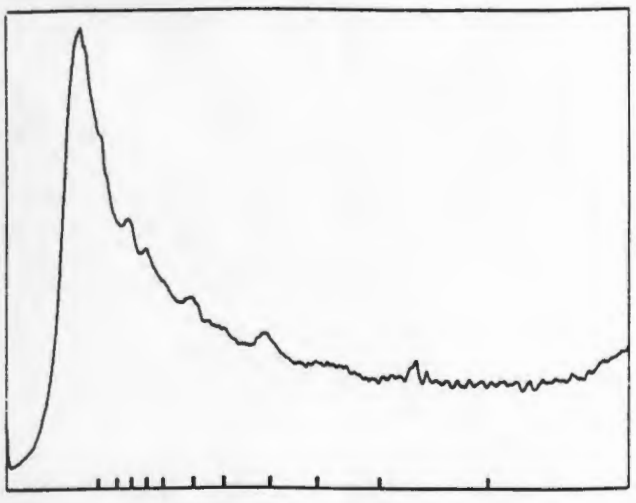
Autoradiographs of the products of DNase 1 digestion of 5'-^{32}P end-labelled control and hybrid core particles. The digestion was stopped at 0, 0.25, 0.5, 1, 1.5, 2, 3 and 5 min (lanes 1-8). The standard (S) is a *Hpa*II digest of pBR 322 labelled by a "fill-in" reaction with the Klenow fragment of *E.coli* DNA Pol 1 using [α-^{32}P] GTP.



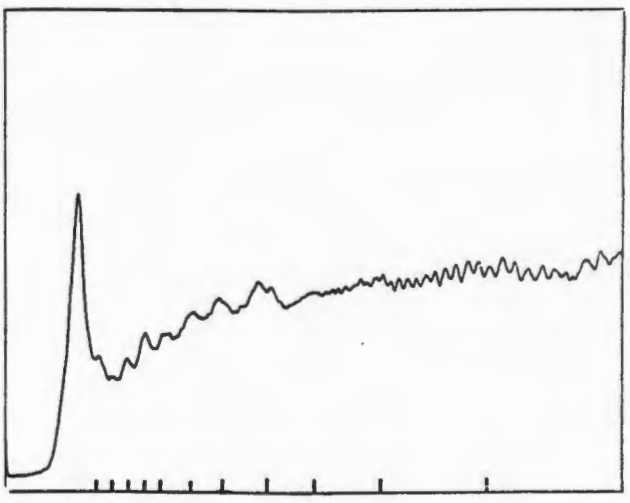
a



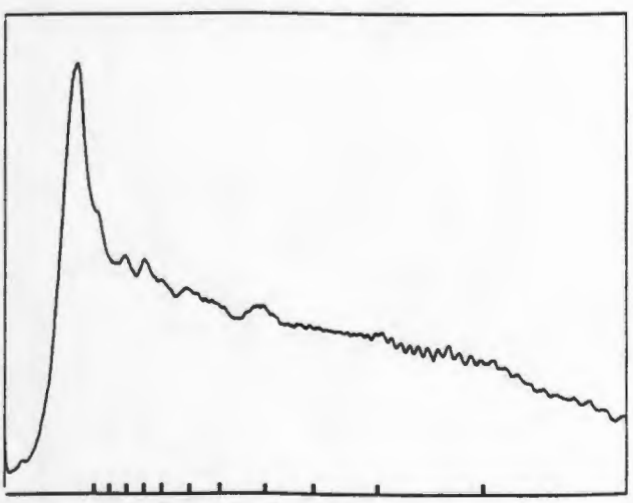
d



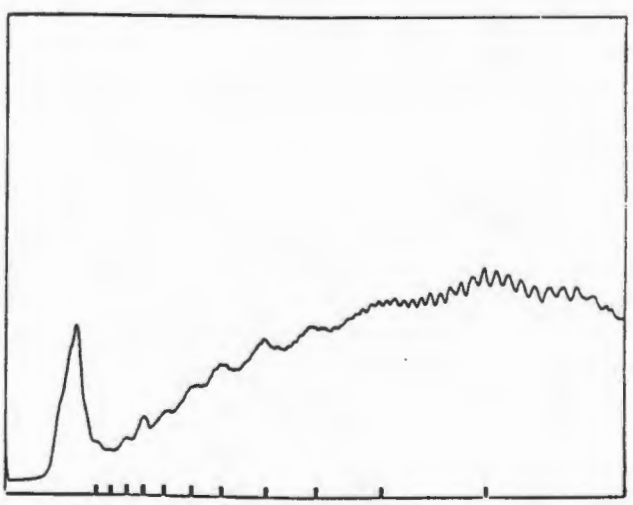
b



e



c



f

Cutting sites (number)

Fig 32.

Densitometric scans of 0.25 minute (a - c) and 2 minute (d - f) DNase 1 digests of control and hybrid core particles shown in figure 31. The scans shown are for uH2A hybrid core particles (a & d), uH2B hybrid core particles (b & e) and control core particles (c & f) .

particle as determined by investigation with both MNase and DNase 1. Comparison of the rate of DNase 1 digestion (figure 33) of the various core particles, however, showed that the ubiquitinated core particles were digested significantly more slowly than control core particles. The order of increasing digestion was uH2A < uH2B < control core particles.

X-ray crystallography (Vijay-Kumar et al. (1987)) and 2D-NMR (Weber et al. (1987)) have both shown that ubiquitin has a tightly folded globular domain consisting of residues 1-72. The remainder of the molecule, residues 73-76, is in a random coil conformation and acts as a linking arm bewteen the globular domain and the histone. Schematically, ubiquitin might be considered as a balloon, the globular domain, attached to a larger structure, the core particle, via a string, the linking arm. The reduced digestion of the ubiquitinated core particles suggest that the ubiquitin attached to histones H2A and H2B in the core particle has considerable lateral mobility thereby causing general rather than specific interference with DNase 1 digestion.

Histone H2B ubiquitination has been shown to be directly linked with ongoing transcription (Davie and Murphy (1990)). Thus inhibition of transcription resulted in a dramatic decrease in uH2B content in a breast cancer cell line. This was reversed upon removal of the transcription inhibitor, dichloro-1-β-D-ribofuranosylbenzimidazole. It was therefore proposed that during transcription the alteration in nucleosome structure allows H2B ubiquitination to occur by exposure of the ubiquitination site. It was further suggested that ubiquitinated histone H2B might impede nucleosome refolding. The results reported above would argue against the latter hypothesis.

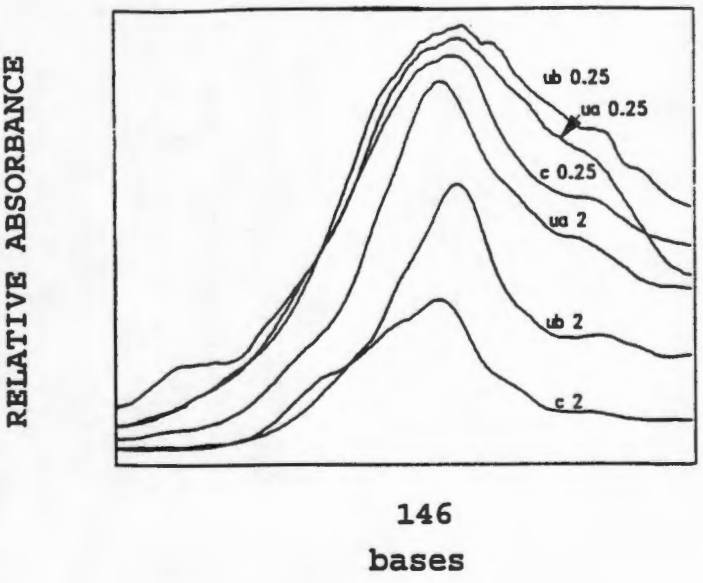


Fig 33.

Analysis of the radioactivity associated with DNA of length 146 bases after 0.25 minutes and after 2 minutes DNase 1 digestion. The partial scans shown are for control (c), hybrid uH2A (ua) and hybrid uH2B (ub) core particles.

CHAPTER 7

CONCLUSIONS

The histone-DNA contacts in the 167 bp and 146 bp core particle are very similar with the 167 bp core particle retaining the essential features of the 146 bp core particle.

Similarities are that the central region of the DNA in the 167 bp core particle is mainly occupied by histones H3 and H4 and the flanking regions by histones H2A and H2B. Histone H3 also binds where the DNA enters and exits the core particle.

Differences are that the 10 bp extensions of the 167 bp core particle are bound by histones H2A and H3. The histone H2A contact at the dyad axis in the 167 bp core particle is diminished relative to that in the 146 bp core particle suggesting that the 167 bp core particle is an intermediate stage in the conformational change that occurs between the the chromatosome and the 146 bp core particle.

A protein which probably binds to the 3' end of the DNA in both core particles has been purified and partially sequenced. The protein appears to be a large histone H2B variant.

Core particles can be assembled onto octamers where histone H2B has been replaced by uH2B. No conformational change in the core particles containing uH2B could be detected by the nucleases MNase and DNase 1. This was surprising as the ubiquitination site of histone H2B is insensitive to trypsin digest when incorporated into the core particle.

CHAPTER 8

MATERIALS AND METHODS

8.1 CORE PARTICLE ISOLATION

8.1.1 Preparation of chicken erythrocyte nuclei

All protocols were carried out at 0-4°C unless stated otherwise. Chicken blood was obtained from "Golden Grove" abattoir, Ottery, Cape Town. 500 mls of blood were collected from the severed jugular vein onto 70 mls of ACD buffer (15 mM citric acid 90 mM tri-sodium citrate 18 mM sodium monophosphate 13 mM D-glucose), sieved through 2 layers of cheesecloth onto ice and transported to the laboratory. Chicken erythrocyte nuclei were prepared immediately. The filtered blood was centrifuged at 5000 g for five minutes and the plasma and white blood cells removed by aspiration. The erythrocyte cells were resuspended in 5 volumes SSC buffer (0.15 M NaCl 0.01 M trisodium citrate, pH 7.2) and recentrifuged at 5000 g for five minutes. The cells were washed a further 2 times. The washed erythrocyte cells were then lysed as described by Drew and Calladine (1987) with modifications. The erythrocyte cells were resuspended in 280 mls of SSC containing 0.1 % (v/v) Nonidet P40 and centrifuged at 10000 g for 30 seconds; the supernatant was discarded. This procedure was repeated until the pellet of nuclei was creamy coloured and free of haemoglobin. The nuclei were then washed twice in 5 volumes SSC and once in 5 volumes buffer A (15 mM Tris-HCl 65 mM NaCl 60 mM KCl 0.15 mM spermidine 0.5 mM spermidine 0.2 mM EGTA 0.2 mM EDTA 5 mM 2-mercaptoethanol 0.01 mM PMSF pH 7.5) (Hewish and Burgoyne (1973)) before storage at -20°C in buffer A containing 50 % (v/v) glycerol.

8.1.2 Isolation of long chromatin and removal of linker histones

15 ml stored nuclei containing 150 to 200 mg DNA were washed three times in buffer A and diluted to 2 mg DNA/ml in this

buffer. $CaCl_2$ was added to 1 mM and the nuclei were digested with 50 units MNase per mg of DNA for 10 minutes at room temperature. Digestion was terminated by addition of EDTA to 5 mM. The digested nuclei were pelleted by centrifugation at 2000 g for 3 minutes, lysed in 10 mls 600 mM NaCl 10 mM Tris-HCl 0.1 mM EDTA pH 7.4 and dialysed against this buffer overnight. The suspension was centrifuged at 10000 g for 10 minutes to pellet nuclear debris and the supernatant was passed over a 1000 x 25 mm Sepharose 4B (Pharmacia) column equilibrated in the same buffer at a flow rate of 12 ml/h to separate the linker histones from the stripped long chromatin (Drew and Calladine (1987)). The complete removal of the linker histones was assayed by SDS PAGE (section 8.6). The leading 80 % of the chromatin peak (monitored at 260 nm) was pooled and dialysed overnight against 5 litres 20 mM NaCL 10 mM Tris-HCl 0.1 mM EDTA pH 7.4 with one change.

8.1.3 Isolation of core particles

The dialysed chromatin was concentrated by ultrafiltration (Amicon PM10 membrane) to 0.75 mg DNA/ml. The MNase digestion to obtain core particles was carried out at room temperature. 500 units of MNase per mg DNA was used for the digestion to 146 bp core particles and 100 units per mg DNA for 167 bp core particles (Lindsey and Thompson (1989)). Trial digestions determined the optimal time period for each new batch of chromatin and enzyme. Digestion was terminated by addition of EDTA to 5 mM. Core particles were isolated by gel filtration using a 1000 x 25 mm Sepharose 6B (Pharmacia) column equilibrated in 100 mM NaCl 10 mM Tris-HCl 0.1 mM EDTA pH 7.4 at a flow rate of 6 ml/h. Protein and DNA contents were determined by SDS PAGE and non-denaturing PAGE respectively (section 8.6).

8.2 CROSSLINKING

8.2.1 Crosslinking of core particles

Crosslinking of purified core particles was carried out essentially as described by Mirzabekov and co-workers (Mirzabekov et al. (1989), Levina et al. (1981)). Core particles (0.5 mg DNA/ml) were methylated with 4.3 mM dimethyl sulfate in 37.5 mM sodium cacodylate 0.1 mM EDTA 0.1 mM EGTA pH 7 at 4°C for 18 hours. The methylated core particles were then exhaustively dialysed against 20 mM KH_2PO_4 1 mM EDTA pH 6.8 and finally into 20 mM KH_2PO_4 2.5 mM EDTA 2.5 mM EGTA pH 6.8. PMSF was added to 0.1 mM and the core particles incubated at 45°C for eight hours. The depurinated crosslinked core particles were then dialysed overnight against 100 mM KH_2PO_4 0.1 mM EDTA 0.1 mM EGTA pH 6.8. The crosslinked core particles were reduced by the addition of 1/20 volume 0.5 M sodium borohydride for 30 minutes; after dialysis against water, the crosslinked core particles were lyophilised.

8.2.1.1 Depletion of uncrosslinked proteins

The removal of uncrosslinked proteins was carried out as described by Mirzabekov et al. (1989) using the cetyltrimethylammonium bromide precipitation procedure. The lyophilised sample was dissolved at 2 mg lyophilate per ml of 1 M NaCl 1 % (w/v) cetyltrimethylammonium bromide 5 M urea 20 mM Tris-HCl 10 mM DTT 2 mM EDTA 0.1 mM PMSF pH 8. 4 volumes of water were added and the solution allowed to precipitate for 15 minutes. The solution was centrifuged at 15000 g for 5 minutes and the supernatant discarded. The pellet was washed once in 0.2 % (w/v) cetyltrimethylammonium bromide and redissolved in 3 M ammonium acetate. After ethanol precipitation, the stripped crosslinked complexes were stored at -20°C.

8.2.1.2 Quantitation of crosslinking

The percentage of protein remaining bound to DNA in the crosslinked complex after removal of free protein (section 8.2.1.1) was quantified using the Coomassie Brilliant Blue G250 binding protocol of McKnight (1977). Chicken histones were used as the standard curve. All assays were carried out in triplicate.

8.2.2 Preparation of crosslinked complexes for electrophoresis

8.2.2.1 Iodination of crosslinked proteins

4 μl of stripped crosslinked complex (5 mg DNA/ml in H_2O), 0.4 mCi $Na^{125}I$ (100 Ci/ml Amersham) and 2 μl 10 % (w/v) SDS were reacted in an Iodogen tube for 10 minutes. (The Iodogen tubes were prepared by evaporating 100 μl 40 mg/ml Iodogen (Pierce) in chloroform in Eppendorf tubes in the dark). After iodination the sample volume was increased to 50 μl by the addition of 1 % (w/v) SDS 1 mM EDTA and the iodinated crosslinked complex was dialysed overnight at room temperature against 2 l of the same buffer (one change). Further dialysis against 1.1 % (w/v) SDS 7.7 M urea 69 mM Tris-HCl pH 6.8 was carried out for 5 hours. 5 μl 10 mM DTT 75 % (v/v) glycerol 1 mg/ml total chicken histones 0.01 % (w/v) bromophenol blue were added and the sample heated at 90°C for 10 minutes before centrifugation at 15000 g for 5 minutes to remove any insoluble material.

8.2.2.2 5'end-labelling of the DNA with [γ ^{32}P] ATP

5'-end-labelling of the DNA was carried out as described by Maniatis et al. (1982). 5 μl of crosslinked complex (5 mg DNA/ml in H_2O) 180 μCi [γ ^{32}P] ATP 20 units T4 polynucleotide kinase (Amersham) 50 mM Tris-HCl 10 mM $MgCl_2$ 5 mM DTT 0.1 spermidine 0.1 mM EDTA pH 7.4 (total volume = 50 μl) were

incubated for 30 minutes at 37°C. The reaction was terminated with the addition of EDTA to 20 mM. Free [γ ^{32}P] ATP was removed on a spin column (Maniatis et al. (1982)).

8.2.2.3 Removal of uncrosslinked DNA

The removal of uncrosslinked DNA was carried out as described Mirzabekov et al. (1989) using hydroxyapatite chromatography. The ^{32}P labelled crosslinked material was ethanol precipitated, resuspended in 30 ml 1 % (w/v) SDS 10 mM DTT 5 mM NaH_2PO_4 pH 6.8 and denatured at 90°C for 10 minutes. The solution was diluted with 9 volumes of 5 mM NaH_2PO_4 pH 6.8 and loaded onto a granulated hydroxyapatite column (50 X 2 mm). The granulated hydroxyapatite was prepared using silica gel (silca gel 60 Merck) as artificial crystallization centers (Mazin and Sulimova (1975)). After 30 minutes, the column was washed with 3.5 ml of 0.1 % (w/v) SDS 5 mM NaH_2PO_4 pH 6.8. Uncrosslinked DNA was eluted with 10 ml 0.1 % (w/v) SDS 150 mM NaH_2PO_4 pH 6.8 and the crosslinked DNA-protein complex eluted with 0.1 % (w/v) SDS 500 mM NaH_2PO_4 pH 6.8. The flow rate was 50 μl/minute and the elution was monitored with a Berthold LB 1200 geiger counter. The eluted crosslinked complex (~1 ml) was dialysed overnight against 1 l 0.1 % (w/v) SDS (one change). The dialysate was ethanol precipitated and resuspended in 1 % (w/v) SDS 10 mM DTT 7 M urea 7.5 % (v/v) glycerol 62.5 mM Tris-HCl 0.01 % (w/v) bromophenol blue pH 6.8. The sample was then heated at 90°C for 10 minutes and centrifuged at 15000 g for 5 minutes to remove any insoluble material.

8.2.3 Two dimensional gel electrophoresis

All electrophoretic and related methodologies were essentially as described by Mirzabekov et al. (1989) with some modifications. The nuclease S1 treatment (section 8.2.3.3.2]) was devised in this laboratory.

8.2.3.1 First dimension PAGE

Both ^{125}I and ^{32}P labelled samples were electrophoresed on a first dimension slab gel (250 x 250 x 0.5 mm). The Laemmli buffer system was used (Laemmli (1970)).

Stacking gel (20 mm length)
6 % (w/v) acrylamide
0.03 % (w/v) N', N' me
thylenediacrylamide
7 M urea
0.125 M Tris-HCl pH 6.8
0.1 % (w/v) SDS

Resolving gel (220 mm length)
22.5 % (w/v) acrylamide
0.1125 % (w/v) N', N' me
thylenediacrylamide
7 M urea
0.375 M Tris-HCl pH 8.8
0.1 % (w/v) SDS

Reservoir buffer
0.1 % (w/v) SDS
215 mM glycine
25 mM Tris-HCl pH 8.3
2.2 mM thioglycollic acid (upper ch
amber)

The gel was electrophoresed at 150 V until the tracking dye reached the bottom of the stacking gel and thereafter for 40 hours at 300 V. In the case of the ^{32}P labelled sample, 11 hours after the bromophenol blue front had passed the bottom of the stacking gel, a ^{32}P labelled synthetic 68 base polydeoxyribonucleotide was loaded onto the top of the stacking gel and electrophoresis was then resumed. 36 nCi of the 68 base polydeoxyribonucleotide in 1 % (w/v) SDS 10 mM DTT

7 M urea 7.5 % (v/v) glycerol 62.5 mM Tris-HCl 0.01 % (w/v) bromophenol blue pH 6.8 were heated at 90°C for 10 minutes prior to loading. (See (sections 8.2.3.2.1 and 8.2.4) for the preparation of 68 base polydeoxyribonucleotide and gel solutions respectively). After electrophoresis, first dimension gel strips with a 5 mm width were excised.

8.2.3.2 Second dimension PAGE of ^{32}P labelled crosslinked complexes

The first dimension strips containing ^{32}P labelled complexes were washed twice in 100 mls 0.1 % (w/v) SDS 0.125 M Tris-HCl pH 6.8 for 10 minutes. The strips were then electrophoresed in slab gels (250 x 250 x 1 mM). The strips were polymerised onto a preformed 6 % (w/v) acrylamide stacking gel (25 mm length without urea) using 6 % (w/v) acrylamide stacking gel solution. The resolving gels (205 mm length) were as above except that the acrylamide (A) and N', N' methylenediacrylamide (D) concentrations were either

	% D (w/v)	% A (w/v)
	12	0.06
or	15	0.075
or	18	0.09.

The reservoir buffer was as for the first dimension (excluding thioglycollic acid) (section 8.2.3.1). 3 mg Pronase (Calbiochem) and 0.75 mg heat denatured DNA fragments (obtained from a DNase 1 digest of chicken erythrocyte nuclei, (section 8.2.3.2.2) were added to 1 ml sample buffer (1 % (w/v) SDS 10 mM DTT 15 % (v/v) glycerol 0.0625 M Tris-HCl 0.01 % (w/v) bromophenol blue pH 6.8) and layered over the gel strip. The pronase was added for digestion of the crosslinked histones within the second dimension stacking gel (Cleveland et al. (1977)) and the DNA fragments were added to size the ^{32}P labelled second dimension spots. The gels were electrophoresd at 7 mA overnight before the current was

increased to 14 mA until the dye front reached the bottom of the gel.

SDS and urea were extracted from the gels by washing first with 70 % (v/v) isopropanol (45 minutes) and then with water (20 minutes). The gels were stained in ethidium bromide (1 mg/ml) for 30 minutes and washed for 10 minutes in glycerol. The positions of the unlabelled DNA fragments from the DNase 1 digest were determined under ultraviolet illumination. The positions were marked with a 2 mm cork borer and the gels dried under vacuum. The gels were then attached to pre-flashed X-ray sheets and three holes were pierced through both the gel and sheet before autoradiography. This allowed exact alignment of the gel with the autoradiogram for sizing the ^{32}P labelled spots using the marked DNA fragments.

8.2.3.2.1 Preparation and ^{32}P end-labelling of the 68 base polydeoxyribonucleotide

The 68 base polydeoxyribonucleotide was synthesised on an Autogen 6500 DNA synthesiser (Genetic Design) and was a gift from Dr. Lino Texeira. It was end-labelled with [γ ^{32}P] ATP exactly as described above (section 8.2.2.2) except that 1 μl 68 base polydeoxyribonucleotide (0.1 mg/ml) was labelled with 140 μCi [γ ^{32}P] ATP using 2 units polynucleotide kinase. Free [γ ^{32}P] ATP was removed on a spin column (Maniatis et al. (1982)) and the end-labelled 68 base polydeoxyribonucleotide ethanol precipitated. After resuspension in water, the dpm (disintegrations per minute)/ml were determined by liquid scintillation counting. The end-labelled 68 base polydeoxyribonucleotide could be stored for 1 month at -70°C.

8.2.3.2.2 DNase 1 digest of chicken erythrocyte nuclei

Chicken erythrocyte nuclei were digested essentially as described by (Noll (1974)). Nuclei were diluted with buffer A to 1.5 mg DNA/ml. $MgCl_2$ was added to 10 mM and the nuclei digested with 200 units DNase 1 (Boehringer Mannheim)/mg DNA

for 5 minutes at 37°C. Digestion was terminated by the addition of EDTA to 20 mM and SDS to 1 % w/v. 20 units Proteinase K (Boehringer Mannheim)/mg DNA were added and the solution left overnight at 37°C. The DNA fragments were extracted twice with phenol, twice with ether and then ethanol precipitated.

8.2.3.3 Second dimension PAGE of ^{125}I labelled crosslinked complexes

8.2.3.3.1 Formic acid/diphenylamine treatment of the first dimension gel strip

The crosslinked DNA was hydrolysed in the first dimension strip according to Burton (1967). The gel strip was washed twice with constant shaking in 30 volumes 66 % (v/v) formic acid for 10 minutes and twice in 66 % (v/v) formic acid 2 % (w/v) diphenylamine for 10 minutes. The strip was then incubated at 70°C for 20 minutes in 66 % (v/v) formic acid 2 % (w/v) diphenylamine. The strip was then washed four times with 66 % (v/v) formic acid for 10 minutes, with water until the pH reached 5.5 and then twice with 0.1 % (w/v) SDS 0.125 M Tris-HCl pH 6.8 for 10 minutes. The strip was heated in this buffer for 1 minute at 100°C and electrophoresed in a slab gel (400 x 350 x 1 mm). The strip was polymerised onto the second dimension gel with 6 % (w/v) acrylamide stacking gel solution and overlayed with 1 ml sample buffer containing 0.2 mg total chicken erythrocyte histones. The stacking and resolving gels were as for the first dimension aside from the length of the resolving gel (350 mm) and the absence of urea. The gel was electrophoresed at 250 V with air cooling until the dye front reached the bottom of the gel. The gel was fixed in 20 % (v/v) methanol 7 % (v/v) acetic acid, dried under vacuum and autoradiographed.

8.2.3.3.2 Nuclease S1 treatment of the first dimension gel strip

The basis for this protocol was the ability of nuclease S1 (from Aspergillus oryzae) to hydrolyse single-stranded DNA in the presence of SDS and urea (Vogt (1973), Zechel and Weber (1977)). The first dimensional strip was polymerised to a slab gel (250 x 120 x 1 mm) with 6 % (w/v) acrylamide stacking gel solution. The preformed stacking gel was 8 % (w/v) acrylamide 0.04 % (w/v) N',N' methylenediacrylamide 0.1 % (w/v) SDS 0.125 M Tris-HCl pH 6.8 and 25 mm in length. The resolving gel was as for the first dimension gel (excluding urea). The strip was overlayed with 1 ml 0.1 % (w/v) SDS 70 mM Tris-HCl 20 % (v/v) glycerol 0.005 % (w/v) bromophenol pH 6.8 containing 0.2 mg total chicken erythrocyte histones and 4000 units nuclease S1 (Boehringer Mannheim) (Vogt (1979)). (One unit of nuclease S1 releases one μg acid soluble deoxynucleotides from denatured DNA in one minute at 37°C). The dye front was electrophoresed 15 mm into the stacking gel at 100 V. The stacking gel was then incubated with continuous shaking in 100 volumes 50 mM sodium acetate 0.1 M NaCl 1 mM $ZnSO_4$ pH 4.3 at 37°C. After 15 minutes incubation, the incubation buffer was replenished. The buffer was replenished a further 3 times with a final incubation period of 40 minutes. The nuclease strip was then washed twice with 0.1 % (w/v) SDS 0.125 M Tris-HCl pH 6.8 for 10 minutes. The strip was heated in this buffer for 1 minute at 100°C and then electrophoresed into a "third" dimension gel (400 x 350 x 1.5 mm). The "third" dimension gel was electrophoresed and processed in an identical manner to that described for the second dimension gel of the formic acid/ diphenylamine treatment.

8.2.4 Preparation of gel solutions

The acrylamide (BDH electran)/N',N' methylenediacrylamide (Merck) stock solutions were prepared with electrophoretic grade reagents. The stock solutions were rolled with 10 %

(w/v) activated charcoal (Merck) for 1 hour, filtered and rolled for a further 45 minutes with 5 % (w/v) Amberlite mixed bed ion exchange resin. After filtration, the stock solutions were stored in the dark. When the gel solutions were made up with urea, the pH was checked and adjusted with HCl if necessary prior to treatment with activated charcoal.

8.2.5 ^{32}P labelling of the crosslink site on proteins

The introduction of a ^{32}P label at the point of crosslink on a protein that has been crosslinked to DNA (section 8.2.1) was carried out essentially as described by Mirzabekov and co-workers (Mirzabekov et al. (1989)).

Crosslinked complex (stripped of uncrosslinked proteins (section 8.2.1.1) was lyophilised and resuspended in 250 μl 70 % (v/v) formic acid 2 % (w/v) diphenylamine at 400 μg DNA/ml and incubated at 70°C for 20 minutes. The solution was diluted with an equal volume of water and extracted 3 times with 500 μl water-saturated ethyl ether. The solution was diluted with 3 volumes water and lyophilised. The lyophilate was washed successively with 100 μl 15 % TCA, 100 μl 100 % acetone 0.02 M HCl and finally with 100 μl 100 % acetone. The washed lyophilate was dissolved in 20 μl 50 mM Tris-HCl 1 mM $MgCl_2$ 0.1 mM $ZnCl_2$ 1 mM spermidine pH 9 containing 0.5 unit calf intestinal alkaline phosphatase and incubated at 50°C for 30 minutes. The sample was cooled to 0°C, precipitated by the addition of TCA to 20 % and then centrifuged at 15000 g for 5 minutes. The pellet was then washed successively with 100 μl 100 % acetone 0.02 M HCl and 100 μl 100 % acetone. The washed pellet was then dissolved in 20 μl 25 mM bicine-NaOH 2 mM CaCl2 10 mM dithiothreitol 1 mM PMSF pH 9 containing 2 μg MNase and incubated for 45 minutes at 37°C. The sample was then precipitated and washed as before and dissolved in 20 μl 50 mM Tris-HCl 10mM $MgCl_2$ 5 mM DTT 0.1 spermidine 0.1 mM EDTA pH 7.4 containing 5 units T4 polynucleotide kinase and 40 μCi [γ ^{32}P] ATP and incubated at 37° for 30 minutes. The labelled

sample was precipitated and washed as above and electrophoresed on SDS PAGE (section 8.6).

8.3 IMMUNOLOGICAL METHODS

8.3.1 Immunization

8.3.1.1 Preparation of immunogen

Ubiquitin was modified for immunization as described by Haas and Bright (1985). 1.9 mg bovine ubiquitin and 7.5 mg bovine γ globulin were dissolved in 1.1 ml 0.1 M PBS pH 7.0 resulting in a molar ratio of 4.6 :1. 40 µl of 3 % (v/v) glutaraldehyde was added in 10 µl aliquots at 10 minute intervals to the solution at room temperature. The crosslinking reaction was allowed to continue at room temperature for a further 30 minutes. SDS PAGE analysis of equal aliquots taken before and after glutaraldehyde treatment showed that no crosslinked complexes were able to enter the stacking gel. The crosslinked complexes were then dialysed against 5 l 0.05 M PBS pH 7.0 (100 mM NaCl 50 mM NaH_2PO_4) overnight, SDS was added to 2 % (w/v) and the complexes were then incubated at 90°C for 10 minutes. After dialysis overnight at room temperature against 2 l 0.2 % (w/v) SDS 10 mM PBS pH 7.0, the complexes were stored frozen in 250 µl aliquots containing 0.2 mg crosslinked ubiquitin.

8.3.1.2 Immunization of rabbits

Rabbits were immunized intradermally at multiple sites on the back with 100 µl aliquots of crosslinked ubiquitin in a 1:1 mixture with complete Freund's adjuvant. A total of 0.2 mg crosslinked ubiquitin was administered per rabbit. Booster injections of 0.2 mg crosslinked ubiquitin in a 1:1 mixture with incomplete Freund's adjuvant were given intramuscularly every two weeks. The rabbits were bled from the marginal ear veins prior to each booster and approximately 10 ml serum prepared. The serum was stored at -20°C and the titre

determined by an antibody capture assay with immobilized uH2A (following section).

8.3.1.3 Immunoassay for determination of serum titre

The immunoassay was carried out on flexible polyvinyl chloride plates. 50 µl purified uH2A (section 8.4.2) (10 µg/ml in 0.1 M PBS pH 7.4) was added to each well and incubated overnight at room temperature. The wells were then blocked with 200 µl 3 % (w/v) BSA 0.1 M PBS pH 7.4 for 3 hours at room temperature. After 2 washes with 0.1 M PBS, 50 µl aliquots of 10 times serial dilutions of the immune serum in 3 % (w/v) BSA 0.1 M TBS pH 7.4 (100 mM NaCl 100 mM Tris-HCl) were incubated for 2 hours at room temperature. The plate was then washed 4 times with 0.1 % (v/v) Tween 20 0.1 M TBS pH 7.4 and incubated with 50 µl aliquots of protein A (0.045 µCi) in 3 % (w/v) BSA 0.1 M TBS pH 7.4 for 2 hours at room temperature. The wells were washed a further 4 times with 0.1 % (v/v) Tween 20 0.1 M TBS pH 7.4 and counted in a Packard 5220 auto-gamma scintillation spectrophotometer.

8.3.2 Immunoassay for determination of antibody specificity

Calf thymus uH2A, H3, H4, H2B and H2A in 5 µl 0.01 M TBS pH 7.4 were independently spotted onto nitrocellulose. The core histones were obtained as a byproduct during the purification procedure for uH2A and uH2B (section 8.4.2) and were free of the ubiquitinated histones. After drying the nitrocellulose blots at 50°C for 1 hour, they were blocked with 3 % (w/v) BSA 0.01 M TBS pH 7.4 overnight at room temperature. After 2 washes of 10 minutes each with 2 % (w/v) BSA 0.01 M TBS pH 7.4, the nitrocellulose blots were incubated with a 1/1000 dil of serum (100 µl/cm^2 nitrocellulose) in the above buffer for 2 hours at room temperature. After 4 washes of 10 minutes each with 0.01 M TBS, the blots were incubated with ^{125}I labelled protein A (0.2 µCi/ml) in 2 % (w/v) BSA 0.01 M TBS pH 7.4 for 1 hour at room temperature and then washed a further 4 times

(10 minutes each) with 0.01 M TBS pH 7.4. The blots were then dried and autoradiographed.

8.3.3 Immunoprecipitation

Immunoprecipitations were carried out as described (Harlow and Lane (1988)) with minor modifications. The substrate for immunoprecipitation was ^{125}I labelled using Iodogen tubes (section 8.2.2.1) and unincorporated ^{125}I was removed by gel exclusion chromatography on a pasteur pipette Sephadex G25 column in 0.01 M TBS pH 7.4. The volume of the ^{125}I labelled substrate was then increased to 100 μl by the addition of 2 % (w/v) BSA 0.01 M TBS pH 7.4. 1 μl serum was added and the solution gently vortexed for 1.5 hours. 10 μl protein A-Sepharose 4B CL (100 mg/ml in 2 % (w/v) BSA 0.01 M TBS pH 7.4) was then added and the solution vortexed for a further hour. The matrix was then precipitated by centrifugation (10000 g, 15 sec) and the supernatant removed by aspiration. The matrix was washed 3 times with 100 volumes 2 % (w/v) BSA 0.01 M TBS pH 7.4 and then twice with 100 volumes 0.01 M TBS pH 7.4. 2 volumes SDS PAGE sample application buffer (section 8.6) was added and the solution incubated at 90°C for ten minutes. The matrix was precipitated by centrifugation and the supernatant analysed by SDS PAGE (section 8.6).

8.3.3.1 Cyanogen bromide activation of Sepharose 4B

The activation of Sepharose 4B was carried out essentially as described in (Lowe (1979)). 2 g washed Sepharose 4B was resuspended in 20 ml H_2O and the pH increased to 10.8 with 5 M NaOH. The suspension was stirred slowly and crystalline solid cyanogen bromide added (200 mg/g Sepharose 4B). The temperature was maintained at 18°C with the addition of crushed ice and the pH at 10.8 by the dropwise addition of 5 M NaOH. The reaction was terminated after 12-15 minutes by the addition of an equal volume of crushed ice; at this point the addition of alkali was no longer necessary. The matrix was initially washed with cold H_2O (400 ml) and then with cold 0.1

M $NaBO_3$ pH 9.0 (400 ml). The ligand (in 0.1 M $NaBO_3$ pH 9.0) was then immediately added to the activated matrix and rolled overnight to achieve coupling. Any remaining activated sites on the matrix were then blocked with 200 mM ethanolamine pH 9.5. The ligand-matrix complex was washed exhaustively with 0.1 M $NaBO_3$ pH 9.0 and then washed with the appropiate buffer. Ligand incorporation was determined by the incorporation of a trace amount of ^{125}I labelled ligand. Incorporation was routinely in the range 90- 95 %.

8.3.3.2 Affinity purification of polyclonal antibodies with ubiquitin-Sepharose 4B

5 mg ubiquitin was conjugated to 1 g cyanogen bromide activated Sepharose 4B (section 8.3.3.1) and washed into 0.1 M PBS pH 7.4. The affinity matrix was suspended in 2 ml anti-uH2A serum containing sodium azide 0.02 % (w/v), rolled overnight and then packed into a column (5 X 1 cm). The column was washed with 40 ml 0.1 M PBS pH 7.4 and bound antibody was eluted with 5 ml 0.1 M glycine pH 2.8 onto 0.5 ml 1 M PBS pH 9.3 (Haas et al. (1985)). The affinity purified antibodies were then dialysed overnight against 50 mM PBS pH 7.4. The dialysate was concentrated by ultrafiltration (Amicon PM10 membrane) to 1 mg IgG/ml (1 mg/ml IgG has A_{280} = 1.3 (Harlow and Lane (1988))) and stored at -20°C.

8.3.3.3 Treatment of polyclonal antibodies with histone H3-Sepharose 4B

2 mg histone H3 was coupled to 1 g cyanogen bromide activated Sepharose 4B (section 8.3.3.1) and washed into 0.1 M PBS. The H3-Sepharose 4B was resuspended in 4.9 ml 2 % (w/v) BSA 0.01 M TBS pH 7.4, 100 μl anti-uH2A serum was added and the suspension rolled overnight. The matrix was precipitated by centrifugation (10000 g, 15 seconds) and the supernatant retained. The matrix was resuspended in 2 % (w/v) BSA 0.01 M TBS pH 7.4 and rolled for a further 5 hours. The matrix was

reprecipitated and the supernatant combined with the previous supernatant.

8.4 PURIFICATION OF uH2A, uH2B AND bH2B

8.4.1 Isolation of calf thymus histones

Calf thymus glands were collected on ice immediately after slaughter and stored at -70°C. After thawing, connective tissue, blood vessels and fat deposits were removed. The approximate volume of the thymus was determined before homogenization in a Waring blender for two minutes in 5 volumes 0.14 M NaCl 0.01 M trisodium citrate 5 mM sodium bisulphite. The homogenate was then filtered through 4 layers of cheesecloth and centrifuged for 10 minutes at 5000 g. The pellet was rehomogenized with a Kinematica Polytron homogeniser in approximately 5 volumes of the above buffer and recentrifuged. The pellet was washed in this manner a further 4 times. The washed nucleoprotein was homogenized in 10 volumes 0.4 M HCl to obtain total acid-extracted histones. After centrifugation at 10000 g for 15 minutes, the supernatant was dialysed against deionised water and lyophilised. The lyophilised total acid-extracted histones were stored at -20°C.

8.4.2 Purification of uH2A, uH2B and bH2B

The purification procedure was identical for uH2A and uH2B. Calf thymus total acid-extracted histones (250 mg) were separated at room temperature on a 1000 x 50 mm BioGel P60 (100-200 mesh) column equilibrated with 50 mM NaCl 20 mM HCl with a flow rate of 40 ml/h (Van der Westhuysen et al. (1972)). uH2B eluted with H2B, uH2A with H2A and H3. The relevant fraction was pooled from 3 column runs and loaded onto a 150 x 15 mm CM 52 cellulose ion exchange column in 6 M urea 50 mM NaCl 50 mM sodium acetate pH 5.4 at room temperature (von Holt et al. (1989)). The bound protein was eluted with a linear gradient of increasing NaCl concentration

from 125 mM to 175 mM in this buffer. The eluant, monitored at 230 nm, was analysed for histone content by SDS PAGE and the fractions containing the ubiquitinated histone pooled. The pooled fractions were dialysed against de-ionised water and lyophilised. The lyophilate was resuspended in 8 M urea 2 mM Tris-HCl 10 % (v/v) 2-mercaptoethanol. After 4 hours the pH was adjusted to pH 2.5 (Thorne et al. (1987)) and the solution chromatographed at room temperature on a 1000 x 25 mm BioGel P60 (100-200 mesh) column equilibrated in 7 M urea 2 mM Tris-HCl, pH 2.2 with a flow rate of 5 ml/h. The elution of pure ubiquitinated histone was determined by SDS PAGE analysis. The fractions were pooled, dialysed against de-ionised water and lyophilised. The lyophilate was stored at -20°C.

The purification of bH2B was as above except a total acid extract of chicken erythrocyte nuclei was used as the source (following section).

8.4.3 Isolation of chicken erythrocyte histones

Total acid-extracted histones were obtained from chicken erythrocyte nuclei (section 8.1.1) essentially as described for calf thymus total acid-extracted histones (section 8.4.1) except the HCl concentration used for extraction was 0.25 M. The histones were chromatographed on a BioGel P60 column (section 8.4.2) and resolved into a H2A fraction, a H2B/H3 fraction and a H4 fraction. Histone H3 was further purified by dimerization with o-iodosobenzoate and chromatography on a 1000 x 25 mm Sephadex G100 column equilibrated in 50 mM NaCl 20 mM HCl with a 6 ml/h flow rate (von Holt and Brandt (1977)).

8.5 RECONSTITUTED CORE PARTICLES

8.5.1 Reconstitution of histone octamers

The reconstitution of histone octamers was carried out essentially as described by von Holt et al. (1989) with some minor modifications. Stoichiometric amounts of the individual

histones were combined with a final protein mass of 2 mg. Urea was added to a concentration of 8 M and 2-mercaptoethanol to 0.3 M. The mixture of histones was then dialysed against 2 M NaCl 10 mM Tris-HCl pH 7.4 overnight. The dialysate was concentrated to 2 mg protein/ml through ultrafiltration (Amicon PM10 membrane) and chromatographed on a 850 x 10 mm Sephacryl S300 column equilibrated in this same buffer with a flow rate of 5 ml/h. An aliquot of each fraction was precipitated with 15 % (w/v) trichloroacetic acid, washed with acetone-HCl and electrophoresed on SDS PAGE. The fractions containing equimolar amounts of the histones (i.e. the histone octamer) were pooled.

Octamers with H2A replaced by uH2A or H2B by uH2B were prepared by the same methodology.

8.5.2 Reconstitution of core particles

Core particles were reconstituted using the poly(glutamic) acid methodology (Retief et al. (1984)). 3.125 mg poly(glutamic) acid (Sigma MW 50000-100000) (10 mg/ml in 10 mM Tris-HCl pH 7.4) was added per mg octamer followed by 1.725 mg long DNA (> 1000 bp) per mg octamer. The DNA was prepared by phenol extraction of long chromatin from a MNase digest of chicken erythrocyte nuclei (section 8.1.2). The reconstitution mixture was then dialysed against 20 mM NaCl 10 mM Tris-HCl 0.1 mM EDTA pH 7.4 overnight. The volume of the dialysate was reduced through ultrafiltration (Amicon PM10 membrane) to approximately 3 ml. MNase digestion to obtain core particles was carried out in 1 mM $CaCl_2$ at 37°C with a MNase concentration of 100 units/ml. The optimal digestion time for each reconstitution was initially determined by trial digestions on 125 μl of the concentrated dialysate. Digestion was terminated by the addition of EDTA to 5 mM. Core particles were purified on a 5-20 % (w/v) sucrose gradient in 100 mM NaCl 10 mM Tris-HCl 0.1 mM EDTA pH 7.4. After ultracentrifucation at 165000 g for 17 hours, the gradients were fractionated into 80 μl fractions. An aliquot of each

fraction in 1 % (w/v) N-lauryl sarcosine was electrophoresed on non denaturing PAGE (section 8.6). The fractions containing only 146 bp DNA were pooled and the histone content determined by SDS PAGE after trichloroacetic acid precipitation.

8.5.2.1 DNase 1 digestion of reconstituted core particles

5'-endlabelling of reconstituted core particles was carried out as described in Maniatis et al. (1982). 180 µl reconstituted core particles (9-11 µg/ml DNA), 40 µCi [γ ^{32}P] ATP and 10 units T4 polynucleotide kinase (Amersham) in a final concentration of 50 mM Tris-HCl 10 mM $MgCl_2$ 5 mM DTT 0.1 mM spermidine 0.1 mM EDTA pH 7.4 with a total volume of 200 µl, were incubated for 30 minutes at 37°C. The reaction was cooled to room temperature and 1 unit DNase 1 (Boehringer Mannheim) added. Aliquots were removed at 0, 15, 30, 60, 90, 120, 180 and 300 seconds. Digestion at each time point was terminated by the addition of an equal volume of phenol. The samples were phenol extracted twice, ether extracted twice and ethanol precipitated. The samples were resuspended in 50 µl 95 % (v/v) formamide 20 mM EDTA 0.05 % (w/v) bromophenol blue 0.05 % (w/v) xylene cyanol and denatured at 90°C for 5 minutes.

Denaturing gel.
12 % (w/v) acrylamide
0.6 % (w/v) N', N' methylenediacrylamide
7 M urea
90 mM Tris-HCl pH 8.3
2.5 mM EDTA

Running buffer
90 mM Tris-HCl pH 8.3
2.5 mM EDTA

5 µl aliquots were electrophoresed on a denaturing gel (400 x 350 x 0.5 mm) at 500 V until the bromophenol blue front was approximately 3 cm from the bottom of the gel. A constant

buffer temperature of 40°C was maintained throughout. The gel was fixed in 5 % (v/v) acetic acid 15 % (v/v) ethanol, dried and autoradiographed.

8.6 Densitometric scanning of autoradiograms

Densitometric scanning was carried out using a laboratory built densitometer utilising a light microscope to collect light for the photocell. This enabled a resolution of less than 0.1 mm to be attained. The light source employed was a 'daylight' fluorescent tube. The densitometer has been determined to be linear over several orders of grey filter.

8.7 Non denaturing and SDS PAGE

The length of DNA associated with the core particles was determined after phenol extraction and ethanol precipitation on non denaturing slab gels (250 x 250 x 1mm) (Maniatis et al. (1982)). The gels contained 5.7 % (w/v) acrylamide 0.28 % (w/v) N',N' methylenediacrylamide and the buffer was 30 mM Tris 36 mM NaH_2PO_4 0.1 mM EDTA pH 7.5. The gel was electrophoresed in this buffer. The samples were loaded in 3 % (v/v) glycerol 0.01 % (w/v) bromophenol blue and electrophoresed at 60V for 16 hours. A *Hpa* II digest of pBR 322 was used as a standard.

Protein content was determined on SDS slab gels (250 x 250 x 1 mm) (Laemmli (1970)). The stacking gel contained 6 % (w/v) acrylamide, 0.03 % (w/v) N',N' methylenediacrylamide 0.1 % (w/v) SDS with 0.125 M Tris-HCl pH 6.8 as the buffer. The resolving gel contained 20 % (w/v) acrylamide 0.1 % (w/v) N',N' methylenediacrylamide 0.1 % (w/v) SDS with 0.375 M Tris-HCl pH 8.8 as the buffer. The reservoir buffer was 0.1 % (w/v) SDS 0.2 M glycine 25 mM Tris-HCl pH 8.3. The samples were loaded in 1 % (w/v) SDS 15 % (v/v) glycerol 0.01 % (w/v) bromophenol blue 0.125 M Tris-HCl pH 6.8, heated at 90°C for 10 minutes and electrophoresed either at 250 V for 3 hours or 150 V for 16 hours.

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